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A. F. Blakslee

ALBERT FRANCIS BLAKESLEE

ALBERT FRANCIS BLAKESLEE was born in Geneseo, New York, on November 9, 1874, the son of FRANCIS DURBIN BLAKESLEE, a Methodist minister and educator, and AUGUSTA MIRANDA (HUBBARD) BLAKESLEE. He died in the Cooley-Dickinson Hospital, Northampton, Massachusetts, on November 16, 1954, one week after his eightieth birthday.

After graduating from Wesleyan University in 1896, BLAKESLEE taught mathematics and science at Montpelier Seminary in Vermont for two years, and at the East Greenwich Academy in Rhode Island for another year, before going to Harvard as a graduate student. During his first year there he held a teaching fellowship in Botany, and for the next two years he served as an instructor at Radcliffe College. The summers of those years he spent at the Biological Laboratory in Cold Spring Harbor as an assistant in Botany, and the following summer he was in Venezuela collecting for the Cryptogamic Herbarium at Harvard. After receiving his Ph.D. degree in 1904 he went to Europe for two years as a Carnegie Institution of Washington Fellow, working chiefly in the laboratory of PROFESSOR GEORG KLEBS at Halle, Germany.

He then returned to Harvard for a year as Instructor in Botany, and in 1907 he accepted the position of Professor of Botany and Director of the Summer School at the Connecticut Agricultural College in Storrs (now the University of Connecticut). On leave from the college during the year 1912–1913, he worked at the Carnegie Institution's Station for Experimental Evolution in Cold Spring Harbor—later to be called the Department of Genetics—and in 1915 he joined the staff as a resident investigator in genetics, filling the position vacated by G. H. SHULL, who had moved to Princeton. He became Assistant Director of the Department in 1923. When C. B. DAVENPORT retired in 1934, BLAKESLEE was appointed Acting Director and, a year later, Director. He remained in that position until his retirement in 1941.

In 1942 he moved to Smith College, in Northampton, where he had been invited to serve for a year as WILLIAM ALLAN NEILSON Research Professor of Botany. The next year he was appointed Visiting Professor. With support received from several sources, he organized the Smith College Genetics Experiment Station; and he and his group carried on productive research there for twelve years.

Two dominant interests, biological research and teaching, were strongly expressed in BLAKESLEE's character. By virtue of innate ability, originality, and perseverance he was able to achieve outstanding success in both activities. As a young boy he had displayed a love of outdoor life coupled with a curiosity that led him to observe natural events and to make all kinds of collections. Later in life these inclinations persisted, and played an important part in determining the scope of his research. The materials he worked with included Mucors, trees, poultry, Rudbeckia, Portulaca, the adzuki bean, Datura, and Verbena. In addition

to a wide variety of genetical, cytological, and biochemical problems in Datura and several other plants, he also investigated the physiology and embryology of Datura. Still other objects of his study were sexuality in fungi; identification of trees in winter; the relation of egg-laying performance to coloration of legs, beak, and vent in chickens; and differences in olfactory and taste acuteness among people. While working with Mucors he accumulated an extensive, world-wide collection of strains; and during the period of his Datura research he was an ardent collector of strains and species of that genus. In Storrs he collected plants of agricultural and horticultural interest for a botanical garden, which he established for the students' benefit.

As a teacher, both at Connecticut and many years later at Smith College, he was very effective in arousing the interest and intellectual curiosity of his students; there are scores of men and women who remember their work with BLAKESLEE as a high spot in their college careers. His presentation of material was vivid and often dramatic. Lecturing on chromosomal types in Datura, he frequently used magnetized models of chromosomes, moving them against a background to demonstrate the various configurations. His demonstrations of taste differences were especially effective. During a lecture he would distribute capsules containing crystals of phenyl thio carbamide, or papers impregnated with that chemical, and ask the members of the audience to taste them. The results provided a conclusive illustration of genetic variability in the human species. At one meeting of the AAAS, he had a voting machine installed in the exhibit hall, so that participants in the test could register whether the chemical was tasteless or bitter.

BLAKESLEE's research career yielded a number of major contributions in the field of biology, and particularly in genetics. He made his first important discovery as a graduate student. Working on taxonomy of Mucors, he found that zygospores appeared only when certain strains were brought together. This observation established heterothallism in fungi, and led to recognition of the fact that these simple organisms reproduce by sexual fusion—a completely new and sensational idea, which has exerted important influence through the years, both in mycology and in genetics.

Because his teaching duties at Storrs left little time for research, BLAKESLEE curtailed and later dropped his work with fungi, and began to study higher plants, one of which was Datura. Moving to Cold Spring Harbor, he brought the Datura with him, as well as several other plants; and, after finding a number of new types in the Datura cultures, he concentrated his experimentation on that organism. Especially frequent among the new types were "mutative variants," which differed from the normal plants by a complex of characters, transmitted collectively and segregating in a very unusual fashion. The explanation of their peculiar behavior was discovered in collaboration with JOHN BELLING, a distinguished cytologist with a brilliant, analytical mind who joined BLAKESLEE's group in 1920 and remained in Cold Spring Harbor until 1927.

The collaborative efforts of these two outstanding scientists laid a foundation

for the solution of many puzzling problems of genetics and opened up a new field in cytogenetic research. They supplied conclusive evidence that extra chromosomes were responsible for the mutative variants in Datura; that any one of the twelve Datura chromosomes might become extra, giving rise to a specific phenotype according to the genes it carried (primary types); that an extra chromosome might also be made up of two similar halves of a chromosome (secondary types), or two halves of different chromosomes (tertiary types). In rapid sucession tetraploid and haploid plants were discovered, and thus a way was disclosed to produce at will triploids and all possible primaries, as well as various tetrasomic types (2n+2).

Under BLAKESLEE's leadership an exhaustive cytogenetic analysis was made of this unique discovery in Datura. At Cold Spring Harbor he had satisfactory greenhouse and field facilities for the plants and also adequate funds to provide for collaborators and assistants—especially during the summer, when as many as 70,000 plants were grown outdoors. Of his main collaborators, JOHN BELLING established the basis for the cytological analyses; DOROTHY BERGNER continued the cytological studies after BELLING's departure for Berkeley; SOPHIE SATIN carried out a portion of the cytological studies and in addition made a thorough analysis of patterns of development in several parts of the Datura flower and plant, by means of chimeras produced by colchicine treatment; and AMOS G. AVERY very effectively supervised the plant culturing and the scoring of various traits. Prominent among BLAKESLEE's summer collaborators were E. W. SINNOTT, who investigated the effects of the extra chromosomes on quantitative characters, and J. T. BUCHHOLZ, who conducted studies of pollen-tube growth and developed a technique for shortening the distance to the ovules by splicing a stigma directly to the top of the ovary, thus facilitating many crosses that could not otherwise have been made.

This concentrated research effort brought forth spectacular results. It contributed greatly toward our understanding of polyploidy, polysomic types, segmental interchange between homologous and non-homologous chromosomes, and chromosomal differences between geographically separated strains or different species of Datura. During the later years, at Smith College, BLAKESLEE's interest turned in the direction of physiological problems and the development of early embryos in culture.

In BLAKESLEE's hands, Datura became one of the most useful of plants for genetical research. Through his efforts, methods of culture were developed that allowed four generations per year to be raised from seed, or a desirable plant to be propagated indefinitely by grafting. Cytological techniques were perfected for the efficient study of chromosomal configurations appearing in crosses within a species or between species. Splicing procedures extended the possibilities for crossing mutant types and, more particularly, different species. Embryo-culturing techniques made it possible, in species crosses, to obtain mature plants of types that otherwise would have perished. During the long period of his Datura research, BLAKESLEE assembled not only a large variety of chromosomal types

but also a collection of 541 gene mutations, 81 of which are located in specific chromosomes.

Although he was very generous about sharing the credit for research accomplishments with his collaborators and assistants, and most of his papers were published under joint authorship, BLAKESLEE adhered strictly to the policy that materials accumulated by him must be used only under his control and in his laboratory. This attitude imposed severe limitations on the general study of Datura, and prevented the development of other groups of investigators working with that plant. Datura research is consequently becoming a lost art. But BLAKESLEE's material still has rich potentialities for the solution of many problems in genetics and speciation, and could be used to good advantage.

Some of BLAKESLEE's energies were devoted to solving a number of organizational problems of general importance to biologists. In 1935, when the *American Journal of Botany* was in financial difficulties, he was chairman of a committee that studied the situation and made recommendations to bring about financial stability. He served as president of the Trustees of *Biological Abstracts* during a time when that journal was in similar difficulties. In the late 1930's he instigated the movement to establish the National Science Fund of the National Academy of Sciences, and worked vigorously in its behalf.

BLAKESLEE was the recipient of many scientific honors and recognitions, both in the United States and abroad, during the long productive period of his life. In addition to receiving seven honorary degrees (San Marcos, Wesleyan, Arkansas, Yale, Delhi, The Sorbonne, and Smith), he was a member of the National Academy of Sciences, the American Philosophical Society, and the American Academy of Arts and Sciences, a foreign member of the Royal Academies of Belgium, Denmark, and Sweden, and an honorary member of nine foreign scientific societies. He was president, at various times, of the AAAS, the American Society of Naturalists, the Botanical Society of America, the Society for the Study of Development and Growth, and the Torrey Botanical Club.

(The author is indebted to PROFESSOR EDMUND W. SINNOTT for the opportunity of referring to the manuscript of his article on DR. BLAKESLEE, prepared recently for the *Biographical Memoirs* of the National Academy of Sciences.)

M. DEMEREC

CORRELATION DUE TO LINKAGE IN CERTAIN WILD PLANTS[1, 2]

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A BIOMETRICAL study of a hybrid swarm in the genus Eucalyptus revealed that for three pairs of measurements the product moment correlation coefficients, r, were greater for the hybrid swarm than for the supposed parental species (CLIFFORD and BINET 1954). These results were unexpected and have led to a consideration of the manner in which linkage may contribute towards correlation.

Assuming that in the parent species the dimensions studied were controlled by several genes, as they appeared to be, some at least are likely to be linked as the haploid chromosome number for Eucalyptus is 11 (DARLINGTON and WYLIE 1956). In general, hybrid swarms such as that under consideration are probably of recent origin, and so it is of interest to determine the possible contribution of genetic linkage to correlation after a finite number of generations following hybridization on a large scale.

In what follows, formulae have been developed whereby the genotype frequencies and magnitude of the correlation coefficients due to linkage may be estimated after n generations. These formulae are based on a simplified population model but do provide a basis for discussion when considering field populations.

Most metrical characters are better represented by continuous than by discrete variables. Current theory holds that the heritable part of such characters is transmitted by several polyallelic loci; nevertheless, as a first approximation, the case of two linked diallelic loci is considered, with a competely dominant allele at each locus and with absence of interference between loci.

Model and notation

The mating system to be considered here is characterised by the following assumptions:

1. All zygotes are monoecious or hermaphrodite.

[1] A preliminary survey of the results contained in this paper was given in a lecture, addressed by the senior author to a meeting of the Australian Genetic Society, held in conjunction with the Session of "Section D" of the August 1955 "A.N.Z.A.A.S." Conference, in Melbourne.

[2] Part of the printing cost of the accompanying figures has been paid by the GALTON AND MENDEL MEMORIAL FUND.

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2. The same proportion, s, of the male and female gametes, produced by every zygote, unite at random with those of the opposite kind, produced by the same zyogote. This proportion, $0 \leqslant s \leqslant 1$, is constant for all generations.
3. The remainder of the gametes mate at random.
4. The generations do not overlap.
5. Selection, mutation and genetic drift are not operating.

This system appears to be the simplest idealization of the one believed to exist in many wild plant species. It contains, as special cases, $s = 0$ (JENNINGS 1916, 1917) and $s = 1$, i.e., pure selfing (ROBBINS 1918). The general case, $0 \leqslant s \leqslant 1$, is treated for a single locus by HALDANE (1924) and for two linked loci, at the genetic equilibrium, by BENNETT and BINET (1956).

The following notation was found convenient: A, a and B, b denote the dominant and recessive alleles at the two loci, and the ten possible genotypes are called T_j, ($j = 1, 2, \ldots . 10$) as set out in Table 1; $p_j^{(n)}$ is the expected relative frequency of T_j in the nth generation. Appropriate linear combinations of these p_j may be formed to represent the corresponding frequencies:

TABLE 1

Notation for the genotypes

j	1	2	3	4	5	6	7	8	9	10
T_j	$\frac{ab}{ab}$	$\frac{ab}{aB}$	$\frac{aB}{aB}$	$\frac{ab}{Ab}$	$\frac{ab}{AB}$	$\frac{aB}{Ab}$	$\frac{aB}{AB}$	$\frac{Ab}{Ab}$	$\frac{Ab}{AB}$	$\frac{AB}{AB}$

i. of allele combinations, $p(a\,b) = p_1 + \frac{1}{2}(p_2 + p_4 + p_5)$ and three similar expressions for $p(a\,B)$, $p(A\,b)$ and $p(A\,B)$,
ii. of alleles, $p(a) = p(a\,b) + p(a\,B)$ and three similar expressions for $p(A)$, $p(b)$ and $p(B)$,
iii. of frequencies of genotypes at the individual loci, $p(a\,a) = p_1 + p_2 + p_3$ and five similar expressions for $p(a\,A)$, $p(A\,A)$, $p(b\,b)$, $p(b\,B)$ and $p(B\,B)$.

It is obvious that, for all values of n and s, these frequencies satisfy consistency conditions such as

$$\begin{aligned}\sum_{j=1}^{10} p_j^{(n)} &= p^{(n)}(a\,b) + p^{(n)}(A\,b) + p^{(n)}(A\,B) + p^{(n)}(a\,B) \\ &= p^{(n)}(a\,a) + p^{(n)}(a\,A) + p^{(n)}(A\,A) \\ &= 1\end{aligned}$$

Furthermore, it follows from assumption (5) that the allele frequencies are independent of n; hence in the rest of this paper, they will be denoted by $p(a)$, etc, omitting the superscript (n).

The probability of recombination, usually called p, is denoted by γ ($0 \leqslant \gamma \leqslant \frac{1}{2}$), with $\gamma = 0$ representing perfect linkage (which, in this context, may be regarded as equivalent to pleiotropy) and $\gamma = \frac{1}{2}$, representing the absence of linkage. The rare cases of $\frac{1}{2} > \gamma \leqslant 1$ are excluded from consideration.

The assigned values of the measurements of both characters are taken as 0, 1, for the recessive and the dominant phenotypes respectively; $\mu_1^{(n)}$ $\mu_2^{(n)}$ $\sigma_{(1,1)}^{(n)}$ $\sigma_{(2,2)}^{(n)}$ and $\sigma_{(1,2)}^{(n)}$ denote the means, variances and covariance of these measurements in the nth generation. The well known expressions for these parameters in terms of the $p_j^{(n)}$ are summarized below:

$$\mu_1 = 1 - p^{(n)}(a\,a);\ \mu_2 = 1 - p^{(n)}(b\,b)$$
$$\sigma_{ii}^{(n)} = \mu_1^{(n)}(1 - \mu_i^{(n)}),\ i = 1,2;\ \sigma_{12}^{(n)} = p_1^{(n)} - (1 - \mu_1^{(n)})(1 - \mu_2^{(n)}) \qquad \text{-----1}$$

Recursion relations and their solutions

For $s = 1$, the $p_j^{(n+1)}$ are linear in the $p_j^{(n)}$, hence the methods of powering the generation matrix are applicable. It is rather straightforward, as the powering of the 10×10 generation-matrix is reducible to that of a 4×4 matrix, with latent roots

$$1,\ \tfrac{1}{2},\ \tfrac{1}{2} - \gamma(1 - \gamma),\ \tfrac{1}{2} - \gamma \qquad \text{-----2}$$

in this order. Completion of the process leads to ROBBINS' results as expected.

For $s = 0$, the $p_j^{(n+1)}$ are of the second degree in the $p_j^{(n)}$. They are uniquely determined by the two constant allele frequencies and by $\omega^{(n)}$, where

$$\omega^{(n)} = p^{(n)}(a\,b) \cdot p^{(n)}(A\,B) - p^{(n)}(a\,B) \cdot p^{(n)}(A\,b)\ .$$

Furthermore,

$$\omega^{(n)} = (1 - \gamma)^n\, \omega^{(0)} \qquad \text{-----3}$$

Thus the $p_j^{(n)}$ can be written rather simply in terms of the constant allele frequencies and of n, γ and $\omega^{(0)}$, as shown by JENNINGS.

For $0 < s < 1$, the recursion relations for the $p_j^{(n+1)}$ in terms of the $p_j^{(n)}$ are linear combinations of those for $s = 1$ and $s = 0$, with s and $1 - s$ as coefficients. These expressions can thus be easily obtained, but as they are very cumbersome for printing they are kept on file in the Editorial Office.

The equilibrium frequencies of the genotypes arising from this process are shown in BENNETT and BINET (1956). There does not appear to be, however, any way for obtaining the genotype frequency directly from the recursion relations for a finite number of generations. Therefore, the following artifice was employed.

The $p_j^{(n)}$, may be regarded as elements of a vector $\mathbf{p}^{(n)}$. A nonsingular linear transformation, $\mathbf{V}\,\mathbf{p}^{(n)} = \mathbf{q}^{(n)}$ has been found for which the $\mathbf{q}_j^{(n)}$ satisfy difference equations that are comparatively easy to solve. $\mathbf{V}$ and $\mathbf{V}^{-1}$ are exhibited in Tables 2 and 2′ respectively. $q_1^{(n)} = 1$, trivially, for all n, while most of the other q_j have a biological interpretation.

$$q_2 = p(a\,a) - p(A\,A);\ q_3 = p(b\,b) - p(B\,B)$$

i.e., they are the surpluses of recessive over dominant homozygotes in the marginal distributions.

$$q_4 = p(a\,a) + p(A\,A);\ q_5 = p(b\,b) + p(B\,B)$$

i.e., the frequencies of all homozygotes in the same distributions.

$$q_7 = p_5 - p_6 \quad ;\quad q_8 = p_5 + p_6$$

i.e., the surplus of the coupled over the repulsed phase double heterozygotes, and the frequency of all double heterozygotes respectively.

TABLE 2

Transformation matrix and its inverse

$\mathbf{V} =$

1	1	1	1	1	1	1	1	1	1
1	1	1	0	0	0	0	−1	−1	−1
1	0	−1	1	0	0	−1	1	0	−1
1	1	1	0	0	0	0	1	1	1
1	0	1	1	0	0	1	1	0	1
2	1	0	1	0	2	1	0	1	2
0	0	0	0	1	−1	0	0	0	0
0	0	0	0	1	1	0	0	0	0
0	1	0	0	0	0	0	0	−1	0
0	0	0	1	0	0	−1	0	0	0

TABLE 2′

$4\mathbf{V}^{-1} =$

−2	1	1	1	1	1	1	1	−1	−1
2	0	0	0	−2	0	0	−2	2	0
0	1	−1	1	1	−1	−1	2	−1	1
2	0	0	−2	0	0	0	−2	0	2
0	0	0	0	0	0	2	2	0	0
0	0	0	0	0	0	−2	2	0	0
2	0	0	−2	0	0	0	−2	0	−2
0	−1	1	1	1	−1	−1	1	1	−1
2	0	0	0	−2	0	0	−2	−2	0
−2	−1	−1	1	1	1	1	1	1	1

The difference equations for the q_j are shown in Table 3, with their solutions in Table 4. These results make it possible to describe the genotype frequencies of the nth generation in terms of a hypothetical 0th generation by expressing the $q_j^{(o)}$ linearly in terms of the $p_j^{(o)}$ (by Table 2), then substituting into the expressions in Table 4, thus obtaining the $q_j^{(n)}$ in terms of the $p_j^{(o)}$, and finally, by substituting these into Table 2, obtaining the $p_j^{(n)}$ in terms of the $p_j^{(o)}$. Concisely, this could be expressed as

$$\mathbf{p}^{(n)} = \mathbf{V}^{-1}\,\mathbf{T}^{(n)}\,\mathbf{V}\,\mathbf{p}^{(o)}$$

where $\mathbf{T}^{(n)}$ denotes, by an extension of the conventional matrix notation, the ten formulae in Table 4, expressing the $q_j^{(n)}$ in terms of the $q_j^{(o)}$.

It was found to be unduly laborious to write down explicitly the ten nonlinear expressions which constitute $\mathbf{V}^{-1}\,\mathbf{T}\,\mathbf{V}$. In any case, these formulae would depend on seven arbitrary parameters, besides the allele frequencies and n, γ and s. Thus only certain special results were extracted from them and these are discussed in the next two sections.

Marginal frequencies and parameters

Concentrating on the first locus, it can be seen from Table 4 that

$$p^{(n)}(a\,a) = \frac{1-\left(\frac{s}{2}\right)^n}{2(2-s)}\{1 + (1-S)\,(p^{(o)}(a\,a) - p^{(o)}(A\,A))^2\}$$

TABLE 3

Recursion relations connecting $q_j^{(n+1)}$ *with* $q_j^{(n)}$

[Note: $q_j = q_j^{(n)}$, $q_j' = q_j^{(n+1)}$]

$$q_i' = q_i \ , (i=1,2,3);$$

$$q_k' = \tfrac{1}{2}\{1+(1-s)q_{k-2}^2+sq_k\}, (k=4,5);$$

$$q_6' = q_6+sq_7;$$

$$q_7' = (1-y-\tfrac{1}{2}s)q_7;$$

$$q_8' = \tfrac{1}{4}\{(1-s)[2(1-q_6-2\sqrt{1-y}\,q_7)+(q_6+2\sqrt{1-y}\,q_7)^2-(q_2^2+q_3^2)] +2s(1-y)q_8\};$$

$$q_m' = \tfrac{1}{2}\{(1-s)[q_{m-7}+(1-q_6+2\sqrt{1-y}\,q_7)q_{12-m}]+sq_m\}, (m=9,10).$$

$$+\left(\frac{s}{2}\right)^n\{p^{(o)}(a\,a)+p^{(o)}(A\,A)\}$$

$$+\frac{1}{2}\{p^{(o)}(a\,a)-p^{(o)}(A\,A)\}$$

It is seen that at an individual locus the asymptotic approach to genetic equilibrium is at the rate $-log_e\,(s/2)$, BENNETT and BINET (1956), and that -----4

$$\mu_1^{(n)} = p(A)\{1+2\frac{1-s}{2-s}p(a)\}+\left(\frac{s}{2}\right)^n\{\frac{1}{2}p^{(o)}(a\,A)-2\frac{1-s}{2-s}p(A)\,p(a)\}\text{-----5}$$

The expression for the second locus is analogous, while $\sigma_{11}^{(n)}$ and $\sigma_{22}^{(n)}$ are obtainable from (1) and (5).

Association of characters

The correlation coefficient, $\rho^{(n)} = \sigma_{12}^{(n)}/\sqrt{\sigma_{11}^{(n)}\,\sigma_{22}^{(n)}}$ -----6

is used as a measure of association of the characters in the *n*th generation.

For $s = 0$,

$$\rho^{(n)} = \omega^{(n)}\frac{2\,p(a)\,p(b)+\omega^{(n)}}{p(a)\,p(b)\sqrt{p(A)\,p\,(B)\,(2-p(A))\,(2-p(B))}} \text{-----7}$$

From (3), it is seen that, as expected, under random mating the association of characters due to linkage tends to disappear at the rate of $log_e\,(1-\gamma)$ -----8

TABLE 4

Formulae expressing $q_j^{(n)}$ *in terms of* $q_j^{(o)}$, n, s *and* y

[Note: $q_j = q_j^{(o)}; q_j' = q_j^{(n)}$;

$$G = 1-y-\tfrac{1}{2}s;\quad S = \tfrac{1}{2}s\{1-2y(1-y)\};$$

$$H = \tfrac{1-s}{4}\{2(1-q_6-\tfrac{2s}{s+2y}q_7)+(q_6+\tfrac{2s}{s+2y}q_7)^2-(q_2^2+q_3^2)\};$$

$$U = \tfrac{2y(1-s)}{s+2y}q_7(q_6+\tfrac{s-2y}{s+2y});\ W=(1-s)(\tfrac{2s}{s+2y}q_7)^2.\ ]$$

$$q_i' = q_i\ ,(i=1,2,3);$$

$$q_k' = \tfrac{1-(\frac{1}{2}s)^n}{2-s}\{1-(1-s)q_{k-2}^2\}+(\tfrac{1}{2}s)^n q_k\ ,(k=4,5);$$

$$q_6' = q_6+\tfrac{2s}{s+2y}(1-G^n)q_7;$$

$$q_7' = G^n q_7;$$

$$q_8' = \tfrac{1-S^n}{1-S}H+\tfrac{G^n-S^n}{G-S}GU+\tfrac{G^{2n}-S^n}{G^2-S}G^2W+S^n q_8;$$

$$q_m' = \tfrac{1-(\frac{1}{2}s)^n}{2-s}(1-s)\{q_{m-7}+(1-q_6-\tfrac{2s}{s+2y}q_7)q_{12-m}\}$$
$$-\{G^n-(\tfrac{1}{2}s)^n\}\tfrac{2Gy(1-s)}{(s+2y)(1-y-s)}q_7 q_{12-m}.$$

When $(1-\gamma)^n$ is small, $\rho^{(n)}$ is nearly linear in $(1-\gamma)^n$; to this order of approximation, Table 5 shows the number of generations needed to obtain $\rho^{(n)} \leqslant 0.01\ \rho^{(o)}$.

For $s = 1$, we get by straightforward but tedious substitution

$$\sigma_{12}^{(n)} = p^{(o)}(a\,b) - p(a)\,p(b)$$
$$+ (p_5^{(o)} - p_6^{(o)})\frac{1}{1+2\gamma}\{\gamma-(\frac{1}{2}-\gamma)^{n+1}\}$$
$$+ (p_5^{(o)} + p_6^{(o)})\{\frac{\gamma^2}{2^{n-1}(3+\gamma)}-\frac{1}{4}(\frac{1}{2}-\gamma(1-\gamma))^n\}$$
$$+ 2^{-n+1}\{p(a)\,p^{(o)}(B\,b)+p(b)\,p^{(o)}(A\,a)\} \qquad \text{-----9}$$

From this expression, and from (6), (1) and (5), substituting $s = 1$, $\rho^{(n)}$ can be found. It is seen that the composition of the 0-th generation has an effect on the association of characters which persists right up to genetic equilibrium. It is also

TABLE 5

The number of generations, n, needed to obtain $\rho^{(n)} \leqslant 0.01\ \rho^{(0)}$ *for different probabilities of recombination, y*

y	0.5	0.45	0.375	0.3	0.25	0.2	0.125	0.05	0.005
n	7	8	10	13	17	21	35	90	918

seen that equilibrium is approached at the rate $-log_e(\frac{1}{2})$ ----- 10
as expected from (2).

For $0 < s < 1$, no manageable formulae could be obtained for σ_{12}. Table 4 shows that the rate of approach to equilibrium is

$$-log_e(max\ \{\frac{s}{2}, 1 - y - \frac{s}{2}\}) \qquad \text{----- 11}$$

which contains, as special cases, the rates found above (see (4), (8) and (10)) and also the $-log_e\ \lambda$ (BENNETT and BINET 1956) representing this rate in one particular aspect of the genotype frequencies. The persistence of the association of characters even up to the state of genetic equilibrium is discussed in the same paper.

DISCUSSION

Expressions (3) and (8) show that the trend $\rho^{(n)} \to 0$ with increasing n, for $s = 0$, may be regarded as the sign of the gradual equilibrium of $p_5^{(n)}$ and $p_6^{(n)}$. Now it ought to be noted that the 0-th generation is a fiction and that the causes underlying $p_5^{(0)} \neq p_6^{(0)}$ are likely to persist in all subsequent generations, although not necessarily with unchanged intensity. Obvious causes that come to mind are selection and mutation, the equilibrium of which is well known to determine the values of the allele frequencies (FISHER 1930). Although these factors were excluded from the model for the sake of simplicity, it is relevant to consider briefly how they might operate to maintain an excess of either the coupled or the repulsed phase. It is rather unlikely that a selective differential would exist between T_5 and T_6, the double heterozygotes in the coupled and repulsed phases respectively, as these genotypes generally give rise to similar phenotypes, although in the case of very tightly linked loci, the situation may well be different. If the loci are so close that pseudoallelism is observed, there may then be different phenotypes associated with heterozygotes whose genes are in the coupled and repulsed phases (LEWIS 1950). However, in the case of loci which show an appreciable amount of recombination, the direct effect of selection would in general speed up the equalization, due to random mating, of $p_5^{(n)}$ and $p_6^{(n)}$.

It should be noted that the selection pressures on the two characters associated with a pair of loci are not necessarily additive. The literature of genetics contains many instances in which mutual alteration of genetic expression may profoundly change the selection pressures operating on particular phenotypes. A striking example occurs in the meal moth, *Ephestia kühniella*, in which a red-eyed mutant is known which shows considerably lowered viability. However, when this gene for red eyes is combined with another gene for transparency of eyes, the double

recessive is as viable as wild type. Similar examples are known in Drosophila, in which double mutant stocks sometimes show increased viability over the component single strains, and may in some instances approach the wild type. It is therefore not difficult to imagine an extreme situation of this kind in which the **A B** and **a b** phenotypes are favored against **A b** and **a B**, resulting in an increased proportion of gametes containing either both dominant or both recessive alleles and, therefore, increasing the value of $| p_5^{(n)} - p_6^{(n)} |$.

Another influence maintaining a nonzero value of this difference could be an increase in the mutation rate at one of the loci, resulting from mutation at the other locus. There is evidence to show that mutation rates are themselves subject to genetic control (e.g., RHOADES 1939; IVES 1950).

It may be noted that in his work on barley, MATHER (1949) found it necessary to postulate a surplus of the coupled over the repulsed phase.

If factors such as selection and mutation operate in this way with sufficient intensity, then linkage can persistently contribute to correlation even under random mating ($s = 0$). If $s > 0$, consideration of equation (9) and of the conclusions of BENNETT and BINET (1956) show that correlation, once caused by some disturbance, i.e., deviation from the idealised model, generating $p_5 \neq p_6$, persists right up to equilibrium, even if thereafter the mating system remains exactly the idealised one here considered. The correlation persists after p_5 and p_6 become equalised and even in the absence of linkage. However, if linkage is present, the correlation has a higher equilibrium value which is attained more slowly.

SUMMARY

A hypothetical, monoecious population is postulated in which a fixed proportion of the male and female gametes produced by every zygote unite at random with those of the opposite kind produced by the same zygote, while the remainder partake in panmictic union.

Formulae are given by which the genotype frequencies in a given generation can be expressed in terms of those in an arbitrary earlier generation.

A discussion of the model leads to the brief consideration of some factors that might operate to maintain an excess of either coupled or repulsed phases. If these factors operate with sufficient intensity, then linkage can persistently contribute to correlation, even under panmixia.

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SEGREGATION OF GENES DETERMINING RED CELL ANTIGENS AT HIGH LEVELS OF INBREEDING IN CHICKENS

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THE considerable diversity of the red cell antigens of chickens, as shown in the work of TODD (1930), coupled with the known polymorphic nature of some of the human blood groups, suggested their usefulness in following some of the genetic consequences of long-continued close inbreeding in chickens. In theory such consequences should include a loss in heterozygosity with an accompanying loss in phenotypic variability, but at the time the work described in this paper was begun, it was recognized that the inbred lines of White Leghorn chickens maintained at Reaseheath were not showing any reduction in variability of egg production, age at maturity, and final body weight, although body weight at first egg and average egg weight had declined in variability. PEASE (1948) put forward data showing this, and tentatively suggested that heterozygotes for some few loci might be superior for the first three characters above, which are components of general fitness to survive. Thus the selection for large family size practised at Reaseheath might be selecting the more heterozygous individuals and perpetuating a high degree of variability in these characters. It later appeared that this approach may have been an oversimplification, for the findings of ROBERTSON and REEVE (1952) indicated that an increase in homozygosity might lead to an increased susceptibility to environmental sources of variation. This suggestion received support from the finding of CLOUGH and COCK (1957) that the variability of body weight was greater in one of the Reaseheath lines than in F_1 progeny of this line and another. Moreover it has been observed that the Reaseheath inbreds, which thrived well under the management conditions at the original station, were more difficult to keep alive and productive when transferred to other stations.

It was not at first supposed that the antigen loci were themselves necessarily directly concerned in the selection for heterozygosis, but rather that they would participate in a general slowing down of the progress towards increased homozygosis expected during inbreeding. They might however be more useful than other loci as markers for such a general effect in that they are not subject to obvious direct selection. Most of the known genes of chickens determine either defects, or obvious structural or coloring features which are fixed in most breeds. Only in the case of the concealed plumage pattern genes of some dominant white breeds is there absence of overt selection, and COCK (1956) has recently used such genes as markers showing a retarded progress towards homozygosis during inbreeding.

Later the antigen genes were shown to be more directly concerned. SHULTZ

and BRILES (1953) showed that their *B* locus was heterozygous in more inbred lines than would have been expected in the absence of selection for heterozygotes. In a production-bred flock artificial selection for high egg production had favoured heterozygotes at the *A* locus, while heterozygotes at the *B* locus appeared to have an advantage over homozygotes in general fitness. GILMOUR (1954) reported that segregation at three loci affecting red cell antigens was continuing in one of the highly inbred Reaseheath lines, amounting to about 50 percent of heterozygotes per locus in each generation, and that the more heterozygous individuals had an advantage in reproductive performance and survival. In a later report GILMOUR (1958) extended these findings to six loci. BRILES (1954) showed that hatchability in three inbred lines increased with the level of heterozygosity at the *B* locus expected in different matings, and that, within line and season, heterozygotes were superior to homozygotes in egg production. BRILES and KRUEGER (1955) and BRILES (1956) reported further data along similar lines. A very wide occurrence of continuing segregation at the *B* locus was reported by BRILES, ALLEN and MILLEN (1957). They found segregation in at least 71 out of 73 closed populations studied. The number of alleles varied from eight in a recently closed population to two in the majority of the more highly inbred lines (inbreeding coefficients above 65 percent).

The present paper reports work on the identification of genes determining red cell antigens in four Reaseheath lines. Attempts were made to identify fixed as well as segregating genes. It is to some extent complementary to the work of BRILES and his collaborators, in that the approach has been to make a detailed study of a small group of very highly inbred lines over a period of years, in an attempt to obtain information on all the antigens present. In a subsequent paper data will be presented on the extent of continuing segregation of these loci in all four lines, and the mechanism of selection leading to such continuance.

The inbred lines

The chickens used were all derived from the inbred lines of White Leghorns maintained by annual brother–sister matings at the Northern Breeding Station, Reaseheath, Cheshire, England. The work at Reaseheath has been described by PEASE (1948) and PEASE and DUDLEY (1954). The lines used, together with the year of the first brother–sister mating, are set out in Table 1. It will be seen that these lines are identical with PEASE's C, I, W and R respectively, but two-letter

TABLE 1

Code letters of the Reaseheath lines used, and the year of the first brother-sister mating

PEASE's code	Present code	Year
C	CH	1933
I	IA	1938
W	WA	1938
R	RE	1940

designations are used here to avoid confusion with the single-letter designations of antigens.

METHODS

The large wing vein which lies just subcutaneously over the muscles on the inner side of the humerus was utilized for bleedings and injections. Blood was withdrawn through a needle of 0.70 mms diameter into a syringe containing sufficient 3.5 percent Trisodium citrate dihydrate solution to make a final concentration of one part citrate and six parts blood. Before withdrawal of the needle the vein was firmly occluded by finger pressure. This, maintained for about 45 seconds, prevented subcutaneous bleeding which otherwise obscured the vein for subsequent work. No sterile precautions were found necessary.

Each testing serum was obtained from a bird which had received several intravenous or occasionally intramuscular injections of washed red cells resuspended in one percent saline. Injections were continued at intervals of two or three days until trial bleedings gave serum of satisfactory titre. Quantity bleedings were then made on the third and fourth days following the last injection. After this time the titres fell, slowly with powerful sera and more rapidly with weak. Citrate was used in these bleedings, so that strictly the "serum" was citrated plasma. It was difficult to obtain a good yield of serum from clotted blood, since chicken clots do not retract well, nor can they be compressed even by protracted centrifuging. The citrated plasma was quite satisfactory, although it usually threw a precipitate, presumably of fibrin, either on inactivation of complement by heating for 30 minutes at 55°C, or following storage, or both. This was centrifuged off and did not affect the titre. All sera were stored without addition of preservative at −20°C. Chickens which did not respond to about eight injections were rested for a month or more and then given a second course. Third or even fourth courses were tried in some instances.

Absorptions were made in small glass tubes by adding thrice-washed packed red cells to inactivated serum, shaking, and allowing to stand at room temperature for half to two hours, followed by reshaking and centrifuging. Weak sera were used undiluted, and were only partially absorbed. They were then titrated in parallel against the absorbing cells and others, and used for testing if some cells reacted in two or three dilutions beyond the tube in which the absorbing cells were negative. This method was used only in some developmental work, and not in routine testing. Powerful sera were diluted according to titre and completely absorbed at this dilution. In the first absorption, very few cells were used (e.g., two drops packed cells to 16 drops serum) since otherwise a meshwork of powerful agglutination often developed from which little serum could be recovered. In the second absorption a half volume of cells was used to one of serum, and in later absorptions the amount of cells was adjusted by experience depending on the titre with control cells in the previous test. Great care was taken to prevent overabsorption, which led to nonspecific weakening of sera.

When sufficient information had accumulated about the inheritance of an-

tigens in an inbred line, injections were designed to obtain sera which were specific for the antigenic products of single alleles, and these sera were then used unabsorbed. They were, however, checked by absorption analysis so that their unitary character—within the one line—could be ensured.

The agglutination tests were made by adding one volume of a thin suspension of washed red cells in one percent saline to one volume of serum in small glass tubes 2″ × ¼″ inside diameter. In developmental work, where numbers of small trial absorptions were being made, the volume used in tests was measured by drawing fluid up to a mark on a fine Pasteur pipette, about 1/5 of a drop. In routine work measurements were made in drops (approximately 1/40 cc). The reactions were shaken and stood at room temperature, shaken again after 15 minutes, and read 1–2 hours later, by which time the negatively-reacting cells had settled into a small button on the bottom of the tube. A second reading was taken 2–4 hours later. With most of the powerful routine sera, the positively-reacting cells settled in clumps widely spread out over the base of the tube. This failure of the clumps to fall into the middle of the bottom of the tube occurred because chicken red cells agglutinated with chicken serum have a tendency to stick to glass, as was reported by Todd (1930). The most powerful class of reaction was that in which the clumps firmly adhered to the glass and could not be brought into suspension by flicking the tube. In less powerful reactions the clumps settled in the manner described but came up into suspension following flicking. In an intermediate class of reaction the small clumps formed a ring near the bottom of the tube, but never the characteristic tight button of the negative unagglutinated cells. The above interpretation of the macroscopic appearance was confirmed in the earlier work by examining some of the resuspended cells or clumps microscopically. A difficulty encountered here was that even unagglutinated cells often clumped nonspecifically after a few moments on the glass microscope slide. This was avoided by using slides very thinly coated with Canada Balsam. Microscopic examination was dispensed with once thorough familiarity was obtained with the characteristics of particular sera, although it was occasionally used with weak sera until they were replaced by more powerful duplicates. With some sera the agglutinated cells did not stick to glass but settled into a small button in the bottom of the tube. Such buttons had at least twice the diameter of the buttons formed by nonagglutinated cells, and always had ragged edges.

Many immune sera were made by injections of blood cells between individuals of the RE line. In addition, with the original object of obtaining information on homozygous antigens, individuals were used which were derived from backcrosses of either RE or WA sires on WA × RE crossbreds. Such individuals, and their further descendants, also gave information on the allelism of certain antigens in the two lines.

The B *locus*

This was the second locus to be identified in the Reaseheath birds and it has been shown to correspond with the *B* locus of Briles, McGibbon and Irwin (1950). A small batch of reagents kindly sent from the United States in 1950 by

Dr. W. E. Briles failed to differentiate Reaseheath birds. Two much larger batches sent in 1956 and 1957 by Dr. Briles' collaborator Courtney P. Allen from the Hy-Line Research Laboratories were successfully used in establishing that certain loci identified in Reaseheath birds corresponded with some of those described by Briles and co-workers, as will be described below.

The *B* locus is considered first here since it illustrates a number of general points not so well shown by the other loci. For purposes of description a historical terminology will be used, but it will be replaced at the end of this section by a more realistic genetic terminology.

The first serum used, 478 anti-B, was obtained by injections of cells from female R1318 into her brother R478. These birds were hatched in 1948 and mated in 1949 to produce a subline of the RE line which was eventually discarded. This serum identified an antigen B which segregated in the RE line as if determined by a single gene *B*. Anti-B serum appeared to be unitary in its antibody content when tested with RE individuals, i.e. absorption with cells of any positive bird completely exhausted it. In addition such absorption exhausted it for cells of individuals from any of the other three lines which reacted with the unabsorbed serum. Absorption of the serum with cells of any individual of the WA line, or of any crossbred individual with one WA parent and one non-B parent from the RE line, left a residual reactivity for B cells of RE individuals but not for cells of any WA or such WA-sired individual. This led to the conclusion that the WA line was homozygous for a portion of the B antigen, designated B_2. The B antigen of the RE line was therefore redesignated B_1B_2 and the serum assumed to contain two antibodies anti-B_1 and anti-B_2.

Antigens allelic to B_1B_2 were not first identified by the use of sera developed within the RE line. Indication of the existence of a second allele at this locus was first obtained during absorption analysis of a multiple serum 1513 produced by injection of cells from an RE female R53 into a WA male W1513. These birds had been mated in 1950 to give crossbred progeny which were backcrossed in 1951 to either a WA sire or the RE sire R478. By means of absorption analysis with cells from the two sets of backcross progeny this serum was found to contain three antibody specificities. The first corresponded with anti-Z and identified an allele already known at the *A* locus and described later. This allele was segregating in RE but was absent from WA which was homozygous for another *A* allele. The second antibody reacted with B_1, the major portion of the already known *B* locus antigen of the RE line. (Anti-B_2 was obviously not made in a WA bird carrying B_2.) The third antibody of serum 1513 identified a new antigen which segregated in the RE line as if determined by an allele of B_1B_2, designated *Y*. All the 50 backcross progeny sired by the RE male R478 carried Y, indicating that this male, already known not to carry B_1B_2, was homozygous for the new antigen. This finding further supported the proposition that the two antigens were determined by allelic genes.

The first definitive anti-Y sera were made by planned injections between individuals of certain backcross families, sired by a WA male, in which Y was

segregating. Absorption analysis of the strongest such serum showed that it was unitary in its antibody content for the RE cells used. Tests of several generations of the RE line with this anti-Y serum showed that it was identifying an antigen determined by an allele of B_1B_2. A slight dosage effect was found with the RE antigens at this locus, in that homozygous B_1B_2 cells reacted slightly more strongly with anti-B_1B_2 than did heterozygous cells B_1B_2/Y. Moreover absorption of this serum with the heterozygous cells left a weak residual reactivity for homozygous cells, but not *vice versa*. Mendelian analysis of matings between the backcross progeny showed that the homozygous gene of the WA line, so far called B_2, was allelic to both the *B* locus genes of the RE line, and they to each other.

Sera for identifying the WA antigen B_2 were made in two ways. Firstly, injections of cells from W1513 into R53 (the parents of the crossbreds already mentioned) gave a serum which reacted with cells of all the WA or WA-sired individuals tested and was completely exhausted by absorption with any such cells. This serum was therefore identifying an antigen or antigens homozygous in all the WA individuals used. Evidence derived from the backcross mating to the RE male and from matings between backcross progeny showed that a single WA antigen was identified by this serum, determined by an allele at the *B* locus, designated *K*. This allele had however already been defined by its previous reactivity as B_2, and so the antigen was tentatively assumed to consist of two antigenic factors K and B_2, the first being the major one, and it was redesignated KB_2 and the gene KB_2.

In further work it was found that the Y antigen of the RE line, but not the B_1B_2 antigen, reacted weakly with anti-K serum. This was taken as showing the existence of two antigenic groups K_1 and K_2 in the WA antigen, one of which—K_2—was associated with Y in the RE antigen. The results of absorption analysis of anti-K confirmed this supposition. Thus the alleles were again redesignated, $K_1K_2B_2$ for the WA allele and YK_2 for the second RE allele.

Other sera for the WA antigen were made by injections between selected backcross progeny matched for *A* locus antigens. For example, injection of cells known from family and serological evidence to carry the two antigens $K_1K_2B_2$ and YK_2 into an individual homozygous for YK_2 produced a serum 564 with reactivity anti-K_1B_2. This was a powerful reagent for the WA *B* locus antigen, and could not be fractionated by absorption with cells of any WA or WA-sired individual.

It was expected that this serum would distinguish the two known *B* locus antigens of the RE line in that it would react with B_1B_2 but not with YK_2. It was found on the contrary that cells of many RE individuals which reacted with anti-Y but not with anti-B_1B_2 did react strongly with 564 anti-K_1B_2. This led to the conclusion that there were two RE antigens containing Y, one reacting with anti-K_1B_2 and designated YK_1, and the other the previously known YK_2 which as expected did not react with anti-K_1B_2. This view was confirmed by the production of separate sera in RE individuals, anti-YK_1 by injection of YK_1 cells into homozygous B_1B_2 individuals, and anti-K_2 by injection of B_1B_2/YK_2 cells into an individual of genotype B_1B_2/YK_1. The anti-K_1B_2 serum no. 564 could be used as a

reagent for B_1B_2 cells after absorption with YK_1 cells, and for YK_1 after absorption with B_1B_2.

A summary of the concept held at this stage of the work was therefore that the RE line carried three allelic antigens at this locus, B_1B_2, YK_1 and YK_2, while the WA line was homozygous for an antigen $K_1K_2B_2$ allelic to these three. This terminology expressed clearly the conclusions derived from work with these two lines only, involving agglutination testing, cross-injections between tested individuals, and absorptions. This concept was essentially the same as that of BRILES, McGIBBON and IRWIN (1950), and that held by American workers on cattle red cell antigens (e.g. STORMONT, OWEN and IRWIN, 1951). The idea was expressed as follows by BRILES, McGIBBON and IRWIN (1950) (p. 635):—"the term *antigen* . . . (refers to) . . . the total antigenic product of an allele, while the term *antigenic factor* . . . (refers to) . . . the 'serological components' of which the antigen appears to be composed". In this notation, B_1, B_2, Y, K_1, K_2 were antigenic factors, making up in various combinations the antigens determined by separate alleles.

It was clear on considering the work recorded above that the antigens B and Y which appeared simple within the one line had to receive a more complex interpretation as soon as a second line was studied. Similarly the simple K antigen of the WA line had to be considered more complex when the RE line was studied. Such reasoning suggested that an explanation based on a small and completely known number of antigenic factors might not be sufficient. This conclusion was in fact confirmed when work was extended to the CH and IA lines.

On testing with all the reagents available, CH cells were found to be of two types, those reacting with reagents for B_1B_2, B_1, Y, YK_1, K_1K_2, K_2, K_1B_2, and those reacting with K_1K_2, K_1B_2, and YK_1 only. Several different reagents of each specificity were used. The second category did not react with anti-K_1B_2, when it was absorbed with YK_1 cells, while the first did. The simplest explanation of these findings would be that there were two alleles, the first consisting of $B_1B_2YK_2$ at least, the second of K_1 at least. No segregation was found with IA cells, all reacting with reagents for B_1B_2, B_1, Y, YK_1, K_2 and K_1B_2, but not with reagents for K_1K_2. These cells might be designated B_1B_2Y, but it would be necessary also to postulate an extra antibody in anti-K_2.

The results of absorption analysis of serum 564 (anti-K_1B_2) necessitated the postulation of yet more antigenic factors in order to maintain an explanation in accord with the concept. Absorption with CH cells of the reactivity $B_1B_2YK_2$ removed the reactivity of this serum for YK_1 cells but not for B_1B_2 cells of the RE line, and not for "B_1B_2Y" cells of the IA line. These results are directly contradictory to the explanation in terms of the discrete antigenic factors so far known. Moreover, absorption with IA cells removed the reactivity for IA cells alone, indicating that these cells carried only parts of the "antigenic factors" B_1B_2Y.

At this stage it was realized that there was nothing to be gained in attempting any longer to maintain the previous concept. It seemed entirely likely that the

complexity of nomenclature would increase each time a new line was studied. Accordingly the hypothesis is tentatively proposed that each line possesses its own characteristic antigens at this locus, and that each such antigen has its own pattern of cross-reactivity with antibodies for the antigens of other lines. It is not necessarily suggested that discrete antigenic factors are not involved in the make-up of the antigenic complexes determined by separate alleles, but rather that the number of such factors is too large for them to be easily sorted out. This point is examined further in the DISCUSSION.

The hypothesis put forward here is essentially the same as that suggested independently by BRILES *et al.* (1957) and therefore their terminology—a modification of the prior terminology of BRILES, MCGIBBON and IRWIN (1950)—is adopted. The distinct antigens at my second locus are accordingly designated with the letter B and each receives also a separate numeral subscript. In order to avoid confusion with the series of separate numerals used by BRILES *et al.* (1957) (B_1 up to B_{21}) it is proposed that numbers prefixed with R be used for antigens of the Reaseheath lines. The corresponding designations are given in Table 2. It will be

TABLE 2

Proposed numbering of B *locus allelic antigens of the Reaseheath lines*

Line	Old	New
RE	B_1B_2	B_{R1}
RE	YK_2	B_{R2}
RE	YK_1	B_{R3}
WA	$K_1K_2B_2$	B_{R4}
WA	"Y"	B_{R5}
CH	"$B_1B_2YK_2$"	B_{R6}
CH	"K_1"	B_{R7}
IA	"$B_1B_2Y(K_2)$"	B_{R8}
IA	"non-Y"	B_{R9}

noted that two further antigens, not mentioned above, are included in this table, one extra for each of the lines WA and IA. The existence of segregation at the *B* locus in the WA line was suspected at the time of the above work, for very weak reactions occurred with some but not all cells when tested with anti-Y. Testing of the latest generation (1957 hatch) has now confirmed this. Segregation is occurring with the anti-K_1B_2 reagent (no. 564), while another antigen identified by a new and more powerful anti-Y reagent is segregating as if allelic. Evidently all the WA birds tested earlier carried the antigen $K_1K_2B_2$, and this led to the erroneous conclusion that the line was isozygous. The particular anti-Y reagent used has a titre of 1:64 for the homologous cells (B_{R2}) against which it was prepared, and a titre of 1:4 for B_{R5} of WA. This indicates that these two antigens probably have only a small part of their make-up in common. Evidence of segregation at the *B* locus in the IA line was obtained in recent tests of the 1957 hatch in which two further anti-Y reagents gave plus or minus reactions with a series

of IA bloods. In this case the titre with reacting IA cells was about the same as with RE line reacting cells.

Evidence that the antigens of the Reaseheath lines described above are likely to be allelic with the antigens of the *B* locus of BRILES *et al.* was obtained by serological means, and the genetical testing required for a rigorous proof has not been possible. Considerable numbers of birds, both from the Reaseheath lines and from other sources, were tested with my reagents and with reagents prepared by BRILES, ALLEN and MILLEN. The pattern of reactions with the American *B* locus reagents followed that with the second locus reagents described above, while the American *A* locus reagents gave a similar pattern to that of the first locus reagents (A and Z of my terminology).

The A *locus*

This was the first locus to be identified in the Reaseheath lines, and its identity with the *A* locus of BRILES, MCGIBBON and IRWIN (1950) has recently been shown, as indicated above.

The first two sera were obtained by injections of cells from female R1169 into male R448, and *vice versa*. These birds were a brother-sister pair hatched in 1947 and mated in 1948 to give progeny which continued the RE line. R448 serum was designated anti-A, and R1169 anti-Z. Of ten progeny tested, nine possessed both antigens A and Z, the tenth only A. The progeny of two brother-sister matings from this family were tested. One mating, of the type AZ × AZ gave five A, five AZ, and one Z progeny. The other mating, AZ × A, gave seven A and eight AZ progeny. The simplest explanation of these findings was that the antigens A and Z were determined by allelic genes *A* and *Z*. The A parent was thus homozygous *A*/*A* and the other three parents were heterozygous *A*/*Z*. All subsequent breeding work has confirmed this interpretation, the totals of progeny from many matings of the type AZ × AZ being close to a 1:2:1 ratio, and of A ×AZ or Z × AZ to the appropriate 1:1 ratios. The one exception is the A/A female which, being the offspring of R448 (Z) and R1169 (A), should have been AZ and not A, if the genetic explanation is correct. We must either suppose a more complex genetic interpretation of the antigenic results, which hardly seems justified in view of all subsequent experience, or postulate another allele which was rapidly lost. Alternatively we may conclude that this bird was mispedigreed or was the offspring of another cock. This conclusion could be unfortunate for the history of the RE line as a true brother-sister mated line, since the rest of the line stems from this bird. This view is perhaps unduly pessimistic, since the other antigens segregating in the subline derived from her supposed full-sibs were found to be identical with those segregating in the main line derived from her, so that the mismating or mispedigreeing, if it did occur, was almost certainly within the RE line. Antigens introduced by a mismating to a bird from one of the other lines would have been easily identified. Antisera to A and Z have been made on many subsequent occasions by appropriate injections, as AZ → Z, AZ → A, A → Z, Z → A. All these have had the same specificity as the originals. Ab-

sorption analysis of the original sera and some of the more recent ones with RE cells has suggested that they are unitary in character. Absorption of the more powerful anti-A sera with AZ cells leaves a very weak residual reaction with A cells, but this is interpreted as a dosage effect, since A cells (A/A) react at a slightly higher titre than AZ cells (A/Z). A similar phenomenon has been found with some anti-Z sera, and with some others, mentioned elsewhere. Genetic evidence from linecrosses and backcrosses has confirmed the above interpretation of the inheritance of A and Z.

All WA and CH cells tested reacted with anti-A but not anti-Z, while IA cells were of reactivity A or AZ. The WA and CH lines may therefore be isozygous at the *A* locus, while the IA line is still segregating. No very definite conclusion has been reached as to the relationship between the A antigens of different lines. All the cells from different lines which react with anti-A reagents have closely similar titres, while no sharp fractionation of reagents is possible in absorption analysis. To take one example, absorption of an anti-A reagent with WA cells leaves no more than a very faint reactivity for homozygous A cells of the RE line. It is thus not technically possible with the present methods to go beyond the conclusion that the antigens of the four different lines defined by their reactivity with anti-A reagents appear to be very closely similar and possibly identical. The same applies to Z. In conformity with the prior terminology the antigens A and Z of the RE line are now designated A_{R1} and A_{R2} respectively.

The third locus

A third segregating locus has been found in the RE line. Most tests have used a single serum 864, designated anti-L, obtained by cross-injection of blood between two progeny of the backcross mating RE × (WA × RE). The donor and recipient were alike in their reactivity with all the *A* and *B* locus reagents then available, except for a very slight difference in titre with serum 53 (original anti-K). This series of injections was one of several made at that time in order to investigate whether this slight difference had any significance, and it was the only one which gave a reactive serum, and that only after a second course of injections.

The antigen L, identified only by its reaction with anti-L, is inherited independently of the *A* and *B* locus antigens. It is segregating in the RE, CH, and WA lines but is not present in IA. This line may therefore be fixed for a non-L allele, or any segregation at this locus may involve other unidentified alleles. L appears to be determined by a single gene as shown by Mendelian analysis of eight generations of the RE line, and of the backcross generations from WA × RE and further descendants of these, previously described. In both cases matings of the type L × non-L have given an approximation to a 1:1 ratio of L and non-L progeny, while L × L matings have fallen into two categories, those giving L and non-L in about 3:1 ratio, and those giving all L. Thus the simplest genetic interpretation is that L is determined by one of a pair of alleles.

This locus has been most difficult to work with since the original serum was

not very strong, and no cross-absorption work has been possible. Moreover no other antigenic factor has been identified which appears allelic to L. Only two out of numerous injections within the RE line of the type L → non-L (with other factors matched) have given L antibody, and in each case this was mixed with another new antibody, which had to be removed by appropriate absorption. Clearly the antibody response to L is difficult to stimulate, and both the original and the new sera were obtained reactive only after a second course of injections. Most of the attempted injection series have now run into third and fourth courses without response. Several attempts have also been made to prepare antisera for the supposed antigen allelic to L by injections of non-L blood into birds which the genetic evidence suggested might be homozygous L/L, (i.e. +/+ → L/L) and of heterozygous L into homozygous L (i.e. L/+ → L/L), but without success.

One possible explanation for the difficulty of producing L sera was that incomplete antibodies might be produced. Apparently negative sera obtained from sets of injections designed to produce L were accordingly tested by the antiglobulin technique. Dr. R. R. A. Coombs kindly provided serum from rabbits which had been injected with chicken plasma, and this was absorbed with pooled chicken cells. The test was performed according to the technique described by Race and Sanger (1954), but without result. Thus incomplete antibodies were not formed. Control tests of the efficacy of the antiglobulin serum were made by using it against cells which had been sensitized with subagglutinating doses of known reactive sera. Powerful agglutination occurred with the antiglobulin serum when the cells had been sensitized with the reactive serum in three or four doubling dilutions beyond the normal titre.

Bloods of numerous birds unrelated to the Reaseheath lines tested with anti-L serum showed sharp segregation into L and non-L categories. These did not correspond with any of the *A* and *B* locus categories obtained by testing with both the author's reagents and those prepared by Briles, Allen and Millen. A few reagents for antigens of the *C* locus (Briles, Briles and Quisenberry 1950), the *D* locus (Briles 1951), and the *E* locus (Briles 1958), made available in the same way, distinguished categories which also did not correspond with the L categories. Injections between these birds gave new anti-L sera. Two series of injections in somewhat inbred Rhode Island Reds (two generations sib-mated) and one series of injections in White Leghorns (also two generations sib-mated) yielded powerful anti-L sera whose reactions paralleled those of the original 864 anti-L serum in tests with both Reaseheath and the above non-Reaseheath birds. The surprising ease with which these powerful sera were obtained in the first injection series in each case contrasts sharply with the difficulty of obtaining even weak anti-L sera in the RE line and the backcross descendants.

Further segregating antigens

A number of further antigens of the red cells have been found segregating in the RE line. Antibodies for the first of these were obtained during attempts to produce antibodies for L by injections of L blood into non-L individuals, with

the powerful antigens of the *A* and *B* loci matched. In one case, serum no. 2180, antibody distinguishing L was obtained, but it was mixed with another antibody and thus the serum required absorption with selected reacting non-L cells to make it specific for L. Following the complementary absorption, with selected L cells, a reagent was obtained for another inherited antigen segregating in the RE line apparently independently of the known antigens. Another series of injections for L produced a serum (no. 2033) which contained weak L antibody ineffective at a dilution of 1:4, and powerful antibody for the same new antigen. Further appropriate series of injections produced specific reagents for the antigen, at first designated M. It was discovered in 1955 among survivors of the 1953 and 1954 hatches, and did not appear in the 1955 hatch. It was therefore impossible to accumulate sufficient data from the RE line to determine its mode of inheritance. Recent tests of birds of the IA line have however yielded further information about M, for it was found to be segregating in this line in parallel with A_{R2}, while the reactor cells also reacted with an American *E* locus reagent. It therefore seems likely that *M* is in fact an *E* locus allele, for it is known (BRILES 1958) that *A* and *E* are associated and probably closely linked. Retests of survivors of the RE line have confirmed that the reactions of this E reagent also parallel M reactions here. The numbers are not sufficient for an accurate test of linkage, but inspection of the test results suggests that the *E* allele identified by anti-M was linked in coupling with A_{R1} in the reacting birds of the RE line, but that the repulsion phase was also present, since the line is now isozygous for A_{R1} and non-*M*.

A further antigen was identified following a series of injections designed to produce an M reagent. This gave a reagent which reacted with all M cells, but also with some but not all non-M cells. This new reactivity was found to concern an antigen which is still segregating in the RE line, designated N. Further reagents specific for N were made by injecting N non-M cells into non-N non-M birds. Study of the 1955, 1956 and 1957 generations suggests that N is determined by a single gene, since matings of the type N × N gave either all N progeny, or N and non-N in a 3:1 ratio.

Further antigens segregating in the RE line were identified by a study of the weak reactions of certain cells with certain powerful sera. Many such sera which gave clear-cut plus and minus reactions when highly diluted reacted with some but not all of the negatively reacting cells when undiluted. In the earlier work this finding was ignored, but it was later thought worth while to examine its significance more thoroughly. The study was facilitated by the fact that the RE line has recently become isozygous at both the *A* and *B* loci. A_{R2} was absent from the 1955 hatch, leaving A_{R1} isozygous. At the *B* locus two alleles have been lost, B_{R2} in 1953 and B_{R1} in 1956. This meant that large numbers of bloods could be tested for weak reactions with reagents for the absent alleles.

Consistent segregation, indicating the existence of a new antigen, was obtained during testing of homozygous B_{R3} bloods of RE individuals with two separate powerful B_{R1} reagents made in backcross progeny. The first, no. 129,

which distinguished B_{R1} clearly at 1:128, reacted with all homozygous B_{R3} bloods when undiluted. Two categories could be distinguished, reactors to 1:8 and reactors to 1:24. A second reagent no. 714 was more powerful, distinguishing B_{R1} at 1:1024, and distinguishing homozygous B_{R3} bloods into categories reacting to 1:16 and 1:48. The strong and weak categories obtained with either reagent coincided. Injection of blood from strong reactors into weak reactors produced specific reagents for an antigen carried by the strong reactors. The results of tests of members of five successive generations of the RE line including several complete families (sire, dam, and progeny) were consistent with the view that this antigen is determined by a single gene. Moreover, in all 196 cases where tests were made with both anti-N and the new reagents, the bloods reacted with one or other or both, strongly suggesting that N and the new antigen are allelic. No dosage effect was observed in these tests. This allelism was confirmed by a study of the family ratios, and these antigens were accordingly designated N_1 and N_2 respectively. The N_1 and N_2 reagents have shown no correspondence in any of their reaction patterns with any others of mine nor with the American reagents of the A, B, C, D or E series.

A further segregating RE antigen was discovered during testing of a reagent produced within the RE line. This reagent (no. 2036) identified A_{R2} at 1:128, but was found to distinguish non-A_{R2} bloods into three categories, nonreactors with the undiluted serum, those reacting to 1:4, and those reacting to 1:12. The nonreactors were used as recipients in injections, some receiving blood from the stronger reactors, others from the weaker reactors. All the sera produced were identical in specificity, distinguishing non-A_{R2} reactors with reagent 2036 (at either strength) from nonreactors. This suggested that an antigen, at first designated Q, was segregating. The reagents made in the above manner were weak and only one of them distinguished bloods into the two different strengths of reaction. A powerful Q reagent was made by reinjecting the original recipient bird (R 2036), after a rest, with cells of one of the reactors. This donor was non-A_{R2} and so the serum should have contained only anti-Q. The expected absence of anti-A_{R2} was confirmed by appropriate tests. With this serum a more clear-cut distinction of the categories of reactors could be obtained. Cells fell into three groups, nonreactors with undiluted serum, those reacting to a titre in the range 1:16 to 1:40, and those reacting at least to 1:128.

A further antigen allelic to Q was discovered accidentally during attempts to produce a strong specific anti-L reagent to replace serum no. 864, stocks of which were almost exhausted. Bird no. R 2180, which had previously produced mixed anti-M + anti-L, was injected with blood from another L individual (non-M, non-N). A reactive serum was obtained which on testing with a panel of known cells was found to identify not L, M or N, but instead a new antigen, designated V, which appeared to stand in an allelic relationship to Q. All bloods tested carried either Q or V or both. The strong reactors with anti-Q did not carry V, the intermediate reactors with anti-Q were also intermediate in their strength of reaction with anti-V, and nonreactors with anti-Q reacted most strongly with

anti-V. The genetic situation is therefore presumably that the three categories are respectively Q/Q, Q/V, and V/V. Tests with American reagents suggested that this locus was identical with the C locus (BRILES, BRILES and QUISENBERRY 1950). One C locus reagent exactly imitated the reactions of anti-Q, including the dosage effect, while another, made in a different line, imitated anti-V. In accordance with the convention previously proposed Q is renamed as C_{R1} and V as C_{R2}. It is worth recording that in this case where the dosage effect was rather marked, the heterozygous cells were variable in their titre with the stronger serum 2036 (anti-C_{R1}), reacting variously in the range 1:16 to 1:40.

Another inherited antigenic property of the red blood cells has been found segregating in the RE line. NAGLER (1942) observed that chicken red cells were agglutinated by suspensions of vaccinia virus grown on chick chorio-allantoic membrane, and that some cells were much less susceptible than others. Later CLARK and NAGLER (1943) found that cells from 55 percent of 78 individuals of various age, sex and breed were strongly agglutinated at a virus dilution of 1:10. They concluded that there were two categories of individuals, and supposed that the distinction had a genetic basis. The mode of inheritance of this character has now been worked out in collaboration with DR. A. C. ALLISON. At his suggestion bloods from all surviving members of the 1953 to 1956 generations of the RE line were tested by him for agglutinability by vaccinia virus. Most cells were strongly agglutinated, but some were not, and it is tentatively suggested from inspection of the pedigrees that the property of non-agglutinability is determined by a single recessive gene. Matings of the type "agglutinable" × "non-agglutinable" gave all "agglutinable" progeny. Brother-sister matings of these progeny gave some "agglutinable" and some "non-agglutinable" progeny, and matings of such "non-agglutinable" sibs gave all "non-agglutinable" progeny. The numbers so far tested are relatively small, and further work is planned to submit the hypothesis of a single recessive gene to a rigorous test. No significant difference in the titre of the reaction was obtained with any of the reacting cells, although some of them were certainly heterozygous. No connection seems apparent between this character and any of the antigen loci described above, and it must be concluded that segregation of yet another locus was continuing at least into the 1956 generation. The further continuance of segregation is not precluded.

Some evidence has been obtained that a further locus was segregating in the RE line, from results of tests of surviving members of the 1953–1956 generations with American reagents for the D locus (BRILES 1951). With four different D locus reagents all bloods reacted alike except for that of one individual of the 1953 generation, which reacted strongly with two reagents for D_1 with which all other bloods did not react, and weakly with two reagents for D_2 with which all other bloods reacted strongly. It is likely that this individual was D_1/D_2 and that the line is now isozygous for D_2. A first series of injections of this blood into other matched individuals has not so far given anti-D_1. This individual belonged to a subline which was not selected for breeding in 1954, and which branched off from the main line in the 1949 generation. In the absence of mutation we may

suppose that the main RE line was segregating at the *D* locus at least until the 1949 generation.

Homozygous antigens

It was expected early in this work that only a few antigen loci might still be segregating in the highly inbred lines, but that many more loci might exist which had become fixed in isozygous condition in different lines. Injections within lines would not produce antibodies for such antigens, but injections between individuals of different lines might be expected to produce antibodies for isozygous antigens in which they differed. Relatively few series of injections between lines were made, but none of them produced evidence that any loci additional to the three then known were fixed in isozygous condition differently in different lines. The WA → RE antiserum no. 53 (anti-K) mentioned above identified antigenic material which was homozygous in the WA donor, which segregated as a unit in backcrosses and their descendants, and which was allelic to the known antigens of the *B* locus. Other sera produced by between-line injections, as of CH → IA, IA → CH, RE → CH, CH → RE etc., have similarly not revealed the existence of different isozygous blocks of antigens in different lines. Most such sera were satisfactory reagents for *B* locus antigens in any line. This finding confirms the view that there exists a number of *B* locus antigens possessing considerable cross-reactivity, and also suggests that these antigens stimulate the production of antibodies more readily than any others present.

Further evidence relating to the supposed blocks of isozygous antigens was obtained from the backcross matings already described. These were designed to produce segregation of any isozygous antigens, thus making possible the manufacture of new antisera by appropriate cross-injections. In many sets of injections between backcross individuals of identical A and B blood types, only one new antiserum was made, that identifying L. Moreover, only two A or B antisera manufactured in such individuals were found to be contaminated with new unknown antibodies. These were found, as reported above, to identify an antigen N_2 which is segregating in both the RE and the WA line. This evidence would seem to suggest that the expected possibly large numbers of isozygous antigens do not exist. Later experience has counselled caution in drawing such a conclusion, for it has been found that while antibodies for A, B, and E antigens are readily made, those for the other known loci are made with great difficulty and are weak.

Number of segregating loci in the RE line

Five of the loci described above, namely *A*, *B*, *C*, *L* and *N*, have been studied in detail and appear to be distinct. The antigens determined by each have been followed through a number of generations and satisfactorily accounted for in terms of the genetic interpretations proposed. Since no allele of *L* was found, special attention was given to the possibility that it might be a third allele at one of the other loci, but study of the records has shown this to be impossible. No matings specifically designed to test for linkage have been set up, but an exami-

nation of relatively small numbers of progeny from some matings in the RE line which seemed to be appropriate revealed no evidence of close linkage between any of these five loci. The possibility that loose linkage may occur cannot be precluded. BRILES (1958) states that the *A* and *E* loci appear to be linked by less than one crossover unit and the fragmentary evidence given above is in accord. SCHEINBERG (1956) found two independent linkage groups (M_2/M_5 and Q_4; S_2/S_3 and O_5) with fairly close linkage (recombination frequencies respectively $5.4 \pm 4.2\%$ and $2.6 \pm 2\%$). In limited tests with two A and two B reagents from BRILES he found a correspondence between Q_4 and B_6, but no others. This would suggest that there is a locus linked with the *B* locus. Altogether three linkage groups may exist, the one including *A*, another probably including *B*, and another (SCHEINBERG's second group). The failure in my work to find segregation of new antigens in backcrosses, and the correspondence of at least four of my loci with four of BRILES' five loci, suggests that the number of antigen loci is limited, so that linkage may well be found in the Reaseheath lines with more work. In summary it may be said that at least five effectively independent loci determining red cell antigens were definitely segregating in the RE line through many years of inbreeding, namely *A-E*, *B*, *C*, *L* and *N*. The vaccinia agglutinability factor was also segregating, and it is likely that this is determined by a sixth locus independent of the others. The *D* locus was probably also segregating until 1953, although this conclusion is based on the different reaction of only one bird. The *A* locus, and with it *E*, was fixed in the 1954 families selected for breeding in 1955, and the *B* locus was fixed in the 1955 families selected for 1956, the third allele having been lost in 1953. In the latest generation tested, that hatched in 1957 from the eighteenth successive brother-sister matings, *C*, *L* and *N* were still segregating. The vaccinia agglutinability factor was segregating at least until 1956, and may still be segregating, although this cannot yet be determined with certainty.

DISCUSSION

The most surprising feature of the work reported above is the finding that all seven of the apparently nonlinked loci identified were still segregating following fourteen generations of brother-sister matings. This strongly suggests that animals heterozygous at these loci were at a selective advantage under the conditions at Reaseheath. Direct evidence of heterozygote superiority and consideration of the way in which it maintained segregation will be presented in a subsequent paper, together with further data on the extent of segregation in the other three lines. Since NEWCOMER (1957) found only five pairs of autosomes in the chicken, corresponding with the five known autosomal linkage groups, it would seem likely that each autosome carries an antigen locus, that the sex chromosome also carries one, and that at least one more case of linkage between these loci must exist besides the known close linkage of *A* and *E*. Thus it is implied that each of the six pairs of chromosomes of the chicken possesses a short segment which tends to continue segregating even under close inbreeding. As regards linkage, it may be noted that SCHEINBERG (1956) found two cases of autosomal linkage of pairs of antigen

loci in the chicken, although apart from a possible involvement of the *B* locus in one of them, nothing is known of their correspondence with the eight loci reported by BRILES and myself.

The *B* locus showed considerable multiple allelism, nine alleles being identified among four highly inbred lines. BRILES *et al.* (1957) similarly found a high degree of antigenic diversity of the *B* alleles, in that 21 distinct antigenic types, each presumably determined by a separate allele, were identified in 12 inbred lines developed from nine different original stocks. Their conclusion that a very large number of *B* alleles exists in chickens is broadly confirmed by the results reported above of cross-reactions, absorptions, and interline immunizations among the four Reaseheath lines. The hypothesis advanced by BRILES *et al.* and by myself, is thus that the *B* locus comprises a large number of multiple alleles each determining one antigen, but that the separate antigens are capable of a very considerable range of cross-reactivity. We may either regard the cross-reactivities as resulting from the fact that each antigen is made up of a large number of antigenic factors eventually identifiable by ordinary serological methods, or suppose them to be comparable with the more discontinuous type of cross-reactivities characteristic for example of the egg albumens of various species of birds and presumably due to chemical characters held in common. In protein antigens these might be particular aminoacids or peptide links, larger combinations of these, or even spatial configurations of part of the molecule (LANDSTEINER 1945). To some extent these two explanations may eventually be found to be equivalent.

As regards the hypothesis of multiple alleles, it should not be forgotten that the similar situation with the human Rh blood group system has been regarded by many workers as capable of a different interpretation. The well known synthesis of FISHER (1944), that three pairs of allelomorphic antigens were involved, was based broadly on the finding that oppositional groupings of reactions could be seen among the complex pattern of reactions characteristic of the Rh system as a whole, and these were thought to be due to the pairs of alleles at three separate but closely linked loci. Confirmatory evidence was adduced from the orders of frequency of the various allelic combinations in populations, and later from the discovery of the postulated crossover combinations in the predicted frequencies (RACE and SANGER, 1954). STORMONT (1955) discussed the linked-gene hypothesis as possibly applicable to the similar complex situation at the *B* locus of cattle, but rejected it because of his failure to find any oppositional groupings among the pattern of reactions. So far as can be seen from Table 3 of BRILES *et al.* (1957), no such sets of oppositional groupings appear in the reactions of the B antigens of chickens. One major difference between human Rh and cattle or chicken B antigens is that the antisera for the postulated separate antigens, C, c, D, d, etc., making up the Rh system rarely if ever cross-react.

The information available for the other loci from the present work is not sufficient to establish the existence of multiple alleles. In an early paper BRILES, MCGIBBON and IRWIN (1950) reported finding nine alleles at the *A* locus, although it would now seem likely that some at least of this complexity was caused

by the various possible combinations of the closely linked *A* and *E* loci. ALLEN (1957) reported the identification of several multiple alleles at each of the loci *A*, *C*, *D* and *E*, although fewer than those found at the *B* locus among the same birds.

SUMMARY

Immune sera made in individuals of the highly inbred RE line of White Leghorn chickens, and in both sets of backcross progeny from a cross of another line (WA) with RE, were used to identify a series of antigens of the red blood cells of chickens. Genetic evidence from inbred and backcross matings showed that the genes determining these antigens occurred at five independent (i.e., not apparently linked) loci segregating in the RE line. In three cases partial dominance was shown by the presence of a dosage effect.

Tests with reagents sent from America showed that three of these loci corresponded with the *A*, *B* and *C* loci already reported by American workers, while the other two (*L*, *N*) were new. A further antigen which had been identified but whose mode of inheritance had not been clearly worked out was shown by such tests to be determined by an allele at the *E* locus of American workers. The probable close linkage of *A* and *E* was confirmed.

A detailed study of the *B* locus showed the presence of nine multiple alleles each determining a separate antigen, distinguished by differing cross-reaction, absorption, and immunization characteristics, among four highly inbred lines of widely different origins.

Two further loci were segregating in the RE line. One of these was *D*, identified only by American reagents, the other a locus determining the agglutinability of chicken red cells by vaccinia virus. In the latter case non-agglutinability appeared to be determined by a single gene recessive to the agglutinability gene.

Thus seven independent loci were segregating in the 1953 hatch of the RE line produced by the fourteenth successive annual brother-sister matings. Attempts were made to identify further antigen loci, which might have become fixed differently in different lines by random drift, by making immunizations between both backcross progeny and individuals of different lines, but without success.

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STUDIES ON SOME POSITION PSEUDOALLELES AT THE WHITE REGION IN DROSOPHILA MELANOGASTER[1]

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POSITION pseudoalleles are noncomplementary mutants which may be divided into separate groups by recombination. Presently found in the literature are two contrasting interpretations of this phenomenon. The first, held by PONTECORVO (1952) maintains that the different pseudoalleles represent alterations at different sites of a single functional unit, the gene. The second, supported by LEWIS (1951, 1955) views the mutants at the different pseudoallelic loci as alterations in different units each of which should be called a gene.

The first interpretation assumes there are several mutation sites on a gene showing position pseudoallelism, and that in each case of mutation the inactivation of the gene results. On this basis, recombination between the mutation sites may be visualized as occurring within the functional unit.

The second interpretation accounts for the noncomplementarity of the mutants at two loci by invoking a position effect involving the products of two genes acting on different steps of a synthetic pathway. From this point of view, recombinant types arise by crossing over between functional units.

As has been pointed out by several investigators, one of the main difficulties in interpreting data obtained from pseudoallelic systems is the inadequate criteria for recognition of a functional unit. BENZER (1957), in his work with bacteriophage, points out the complexity of a functional unit and the difficulties involved in defining it in terms of recombination groups. BENZER's work, along with that of DEMEREC (1955) and co-workers (for review see HARTMAN 1957) using transduction in Salmonella, indicates that the unit of function in these microorganisms may be subdivided by recombination. Whether the recombinational phenomenon recorded in microorganisms is the equivalent of that found in Drosophila is at present a matter of conjecture.

From work with *Drosophila melanogaster* it is clear that the white region is a complex pseudoallelic system. LEWIS (1952) has shown that apricot (w^a) and white–1 (w^1) occupy two loci in this region and may be considered position pseudoalleles. The work of MACKENDRICK and PONTECORVO (1952) supports this interpretation. LEWIS (1956) has also described the position of another gene, white-spotted, (w^{sp}), which may be considered as occupying a third locus in the white region. The loci represented by these three mutants, apricot, white-1 and white-spotted, are located, from left to right in the order named, at about 1.5

[1] This study was begun while the author held a postdoctoral fellowship from the American Cancer Society and is presently being supported by U. S. Public Health Grant C-3648.

on the X chromosome. The work presented here gives evidence that another locus, occupied by Brownex, (w^{Bwx}), lies to the left of apricot. Unexpected crossover types also recorded in this study serve to emphasize again the difficulties involved in the definition of the functional units in this system.

MATERIALS AND METHODS

The establishment of the spatial relationships of the mutants within this pseudoallelic series is based entirely on the recovery of exceptional (nonparental eye-color) offspring from females heterozygous for the pseudoalleles being tested and for appropriate marker genes located on either side of the white region. The markers employed were yellow (y, 0.0; yellow body and bristles); yellow-2 (y^2, dominant to y; yellow body and black bristles); scute (sc, 0.0+; missing scutellar bristles); split (spl, 3.0+; split bristles and rough eyes); echinus (ec, 5.5; enlarged eye facets); singed-3 (sn^3, 21.0; gnarled bristles). The pseudoalleles studied were: apricot, (w^a); blood, (w^{bl}); Brownex, (w^{Bwx}); buff, (w^{bf}); honey, (w^h); white-1, (w^1). Descriptions of all these mutants with the exception of Brownex may be found in BRIDGES and BREHME (1944). Brownex was described by MOSSIGE (1953) as a member of the white series which in heterozygous females, w^{Bwx}/w^+, exhibits a slight dominant effect. This is easily classifiable when such females are made homozygous for the autosomal eye color scarlet. MOSSIGE also reports that crossing over is reduced in the neighborhood of Brownex, with 0.4 map units between scute and Brownex and 1.0 units between Brownex and facet. Crossover analyses done in this laboratory are in close agreement with these figures. Examination of the salivary gland chromosomes shows nothing unusual, although in general the white region is difficult to analyse.

The experimental procedure generally followed was to obtain virgin females heterozygous for two of the pseudoalleles and marker genes located on either side of the white region, and mate them to males carrying $y\ w\ spl\ sn^3$. The females were also made heterozygous for the second chromosome rearrangement *SM1* (LEWIS and MISLOVE 1953), carrying the markers aristaless (al), Curly (Cy), and speck-2 (sp^2), and the third chromosome rearrangement Ultrabithorax-130 (Lewis 1952) which is associated with the dominant marker Ultrabithorax (Ubx^{130}) and carries ebony-sooty (e^s). Such an autosomal constitution is known to be effective in causing an increase in the amount of crossing over in the X chromosome. The offspring resulting from this type of mating were examined for eye colors which differed from either of the pseudoalleles carried by the heterozygous female.

RESULTS

The crosses and any exceptional offspring recovered from each are given in Table 1. There are several results of interest. First, the appearance of y^2w^+ individuals from cross #1 indicates that the locus occupied by Brownex lies to the left of apricot. This may be questioned however since there also appear

TABLE 1

Exceptional recombinant types recovered from heterozygous females

Cross number	Heterozygous female	Exceptional recombinant types recovered	Number	Total offspring examined
1	$+ \quad w^{Bwx} \quad + \quad +$	$y^2 \quad + \quad + \quad +$	3	
		$+ \quad + \quad + \quad +$*	1	43,442
	$y^2 \quad w^a \quad spl \quad ec$	$y^2 \quad w \quad + \quad +$	6	
2	$+ \quad w^{bf} \quad + \quad +$	$+ \quad + \quad spl \quad ec$	1	11,000
	$y^2 \quad w^{Bwx} \quad spl \quad ec$	$+ \quad w \quad spl \quad ec$	2	
3	$+ \quad w^h \quad + \quad +$	$+ \quad + \quad spl \quad ec$	2	14,223
	$y^2 \quad w^{Bwx} \quad spl \quad ec$	$+ \quad w \quad spl \quad ec$	1	
4	$y \quad w^1 \quad spl \quad sn^3$	$y \quad + \quad + \quad +$	2	9,000
	$+ \quad w^{Bwx} \quad + \quad +$			
5	$+ \quad w^{bl} \quad + \quad +$	None		50,250
	$y^2 \quad w^{Bwx} \quad spl \quad ec$			
6	$+ \quad w^{bl} \quad + \quad +$	None		11,909
	$y^2 \quad w^a \quad spl \quad ec$			
7	$+ \quad w^{bl} \quad + \quad +$	None†		76,684
	$y^2 \quad w^{bf} \quad spl \quad +$			
8	$+ \quad w^{bf} \quad + \quad +$	$+ \quad w \quad spl \quad ec$	4	43,025
	$y^2 \quad w^a \quad spl \quad ec$	$y^2 \quad w \quad + \quad +$	4	
9	$+ \quad w^h \quad + \quad +$	$+ \quad + \quad spl \quad ec$	4	13,425
	$y^2 \quad w^a \quad spl \quad ec$	$y^2 \quad w \quad + \quad +$	1	
			Total	196,375

* Single male carrying Ubx^{130}
† For exceptional types not associated with crossing over see JUDD (1957).

y^2w and y^+w^+ classes. The latter of these does not show recombination of the markers on either side of white and may be explained either as a double cross over or as a reversion of Brownex to wild type. It is felt that this single male is not the result of contamination since he carried the third chromosome rearrangement marked with the dominant Ubx^{130}. The y^2w class, on the other hand, presents a problem since it arises from crossing over in the same direction as the y^2w^+ class. In an effort to resolve this complication, attached-X chromosomes heterozygous for apricot and Brownex and suitable markers were synthesized

after the method of MULLER (1936). Attached-X females of the constitution shown in Figure 1 were made heterozygous for the *SM1* and Ubx^{130} rearrangements and mated individually to $y\ w\ spl\ sn^3$ males. The progeny from the females which proved to be of the type shown in Figure 1 were examined for individuals showing exceptional eye-color phenotypes. Among approximately 22,000 offspring examined, four females with wild type (red) eye-color appeared. Phenotypically two were $\mathbf{y^2\ sn^3}$, resulting from the crossover designated as (b) in Figure 1; one was **ec sn**^{3}, which could arise as crossovers at points (a) and (c) or at points (b) and (c) as shown in Figure 1; a fourth was the result of a reciprocal crossover shown at point (a) in Figure 1, and phenotypically was **sn**^{3}. Since the distance from the marked region to the centromere of these X chromosomes is greater than 50 units, the two homozygous classes are rather frequent and the genetic constitution of each female could be readily determined by examining

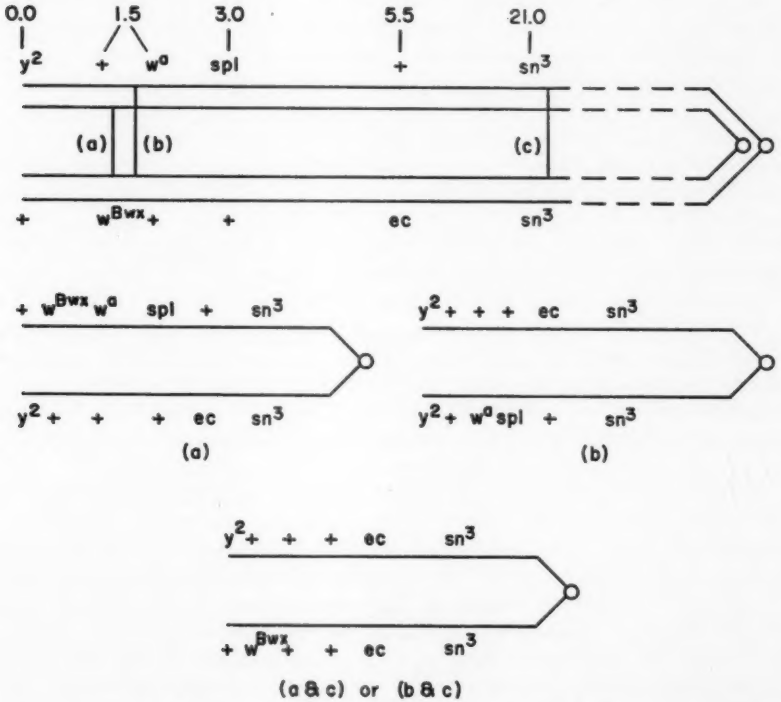

FIGURE 1.—The upper diagram shows the attached-X constitution of the parental females from which crossovers between w^a and w^{Bwx} were detected. Below are shown the three resultant crossover classes which arose from crossing over at point (a) (reciprocal), point (b) (nonreciprocal) and at points (a) and (c) or (b) and (c).

her offspring. Each of the four exceptional females proved to be of the constitution predicted from the crossover types shown in Figure 1.

Examination of the offspring from the female which arose by the reciprocal crossover, (point a), showed that the homozygous double mutant, $w^{Bwx}w^a$, is very similar to w^a in phenotype. On aging, however, the $w^{Bwx}w^a$ darkens to a brownish-pink color while w^a does not. Furthermore, $w^{Bwx}w^a/w^+$ shows the dominant effect exhibited by Brownex; this has not been confirmed by testing with scarlet however. The similarity between the phenotypes of the double mutant and apricot accounts for the fact that the double mutant was not recognized as an exceptional type in the experiments using free-X chromosomes.

Attached-X females homozygous for w^{Bwx} w^a *spl* sn^3 were used to establish a stock culture, and from this a detachment was obtained using the T(1;4) B^S duplication. Females of constitution w^{Bwx} w^a *spl* sn^3/y *sc; SM1;* Ubx^{130} were then synthesized and mated to *y w spl* sn^3 males. Among the progeny of these heterozygous females there appeared a female which was Brownex in phenotype and proved to be w^{Bwx}/y *w spl* sn^3. This gives proof of the existence of the mutant Brownex in the chromosome resulting from the recovered reciprocal crossover, and justifies the conclusion that Brownex occupies a separate locus at the left of apricot.

The use of the attached-X chromosomes does not clarify the origin of the y^2 *w* class obtained from the w^{Bwx}/y^2 w^a *spl ec* free-X heterozygotes. It may well be that this class would not be recognized in the attached-X, since w/w^{Bwx} is very similar to w^a/w^{Bwx} in phenotype and w/w^a cannot always be separated from w^a/w^a; thus, the white class would be recognized only if its complementary crossover type is markedly different from apricot or Brownex in phenotype.

In an attempt to define the exceptional white type, which for brevity will be referred to as w^{72}, several crosses were made with it. These crosses are given in Table 2. It is of interest to note that w^{72} does not cross over with white-1, apricot, or Brownex, though in the latter case the number of offspring examined is not large enough to rule out the possibility that recombination could occur. Furthermore, w^{72} apparently does not represent a double mutant type since no exceptional individuals were recovered from w^+/w^{72} heterozygotes.

Further examination of Table 1 will show that exceptional offspring with white phenotype are recovered from several crosses other than the w^{Bwx}/w^a combination. Crosses numbers 2 and 3 in Table 1 involving Brownex and either honey or buff also give a white class which arises from crossing over in the same direction as the wild type exceptions. These pseudoallelic combinations have not yet been placed in attached-X chromosomes nor have the exceptional types been studied to any extent. Cross number 8 involving apricot and buff offers some results which are also of interest. In this case, only exceptions with white phenotype have been found (w^{86a} and w^{86b}). From the recombination of the markers, it appears that the two may be complementary types. It was considered possible that one of them might prove to be the double mutant, that is, having both apricot and buff together in the same chromosome. To test this assumption, both of

the white types, $y^+ w^{86a}$ *spl ec* and $y^2 w^{86b}$, were crossed to wild type and to white-1 to find whether the original mutants, w^a and w^{bf}, could be recovered from one of them. These crosses and the results are given in Table 2. Neither w^{86a} nor w^{86b} proved to be the double mutant, and the origin of both types remains to be determined.

Early in the course of this investigation, the occurrence of the exceptional white type seemed to be associated with those crosses which involved the mutant Brownex. Working on the assumption that Brownex might represent changes at

TABLE 2

Exceptional recombinant types recovered from heterozygous females

Cross number	Heterozygous female	Exceptional types recovered	Total offspring examined
10	y^2 w^{72*} + + / + w^+ + +	None	19,912
11	y^2 w^{72} + + / *y* w^1 *spl* sn^3	None	12,996
12	y^2 w^{72} + + / y^2 w^a *spl ec*	None	25,888
13	y^2 w^{72} + + / + w^{Bwx} *spl ec*	None	5,402
14	+ $w^{86a\dagger}$ *spl ec* / + w^+ + +	None	14,707
15	y^2 $w^{86b\dagger}$ + + / + w^+ + +	None	10,030
16	+ w^{86a} *spl ec* / *y* w^1 + *ec*	None	24,602
17	y^2 w^{86b} + + / *y* w^1 *spl* sn^3	None	7,144
18	y^2 w^{Bwx} *spl ec* / + w^+ + +	None	26,177
		Total	146,858

* Derived from cross number 1, Table 1.
† Derived from cross number 8, Table 1.

two points located very close together in the X chromosome, offspring from $y^2\ w^{Bwz}\ spl\ ec/w^+$ females were examined. If Brownex is a double mutant, exceptional offspring should also arise from this heterozygous combination. As is shown in cross number 18, Table 2, examination of 26,177 individuals from such a cross failed to yield any exceptional types. The results of this cross also indicate that the derived white class is not the result of unequal crossing over, for if it were, it should arise with comparable frequency from any of several heterozygous combinations tested, including w^{Bwz}/w^+.

The unusual nature of some of the recombinational events which apparently give origin to the recovered exceptional offspring is further emphasized by spatial relationships assumed by the four mutants, apricot, blood, Brownex and buff. Table 1 shows that Brownex-buff and Brownex-apricot combinations give rise to essentially the same classes of exceptions, while apricot-buff heterozygotes give the two unusual white types discussed previously. Blood, on the other hand, does not show recombination with any of these three. It is possible that blood is a small rearrangement in the region of white, but this is not likely since work now in progress shows that blood does recombine with eosin, and that its locus lies to the left of eosin.

The only exceptional types which have been rigorously tested to eliminate the possibility of modifiers are those which arose from the w^a/w^{Bwz} and w^a/w^{bf} females. Stocks of each of the exceptions were established, however, and it can be stated that if modifiers are responsible for the unusual results, they almost always appear in those gametes showing crossing over in the y-spl region of the X chromosome. The appearance of exceptional types not associated with crossing over reported previously (JUDD 1957) from w^{bf}/w^{bl} heterozygotes are presently under study.

An analysis of the wild type exceptions derived from w^a/w^{Bwz} females is now under way. Several of them have now been placed in combination with the mutant zeste, (z), (GANS 1953) and in each case their action with respect to z is indistinguishable from the w^+ derived from the Oregon-R strain. It should be noted however that neither apricot nor Brownex show a suppressor effect in combination with zeste.

DISCUSSION

As more is learned about gene action, it becomes increasingly clear that observable phenotypic effects assigned to a locus may be the result of gene action at any one of several levels. To equate phenotypic change with any particular change in the genetic structure may, in specific cases, be impossible with our present knowledge. The magnitude of a genetic change which will be registered as a change of function may depend entirely on the level at which function is measured. The detection of mutation and thus the definition of the unit of genetic change is of course directly dependent on this measure of function. Similarly, the definition of the unit which can not be further subdivided by recombination

is dependent on the recognition of phenotypic change. In other words, the genetic unit can be properly delimited only when phenotype can be measured at the same level as genotype. From this point of view it is not surprising to find that position pseudoalleles present a unit of function which is considerably different from the unit of recombination.

In the white region of the X chromosome of *Drosophila melanogaster* there are three or four points between which crossing over may be detected. Within this region, which may be defined by the noncomplementarity of the eye-color mutants localized in this part of the X chromosome, there may be several undetected or only partially detected sites which can show recombination with each other or with those sites already known. Whether the $y^2\ w^{72}$ class derived from $y^2\ w^a\ spl\ ec/w^{Bwx}$ females and either or both of the white classes arising from $y^2\ w^a\ spl\ ec/w^{bf}$ females can be explained on the basis of additional loci is not settled.

It is possible to consider the mutant Brownex as a small rearrangement, possibly an insertional duplication for all or part of the white region. It is still difficult however to account for the exceptional white types. Possibly they also are small rearrangements which will not recombine with some or all of the mutants of the white series. It is hoped that this possibility may be subjected to critical test. Even if Brownex is considered to be abnormal with regard to the number and/or arrangement of the components comprising the white region, the data obtained regarding its relationships in space and function to the other mutants of the white series are of value. Whether these data may be explained in terms of conventional recombination events or whether a reconsideration of generally accepted views is called for must necessarily await further work with other members of the white series, other suitable pseudoallelic loci and analysis of the resultant exceptional types.

SUMMARY

Recovered recombination classes from females heterozygous for the mutants Brownex and apricot show that Brownex occupies a separate locus at the left of apricot. The possibility that Brownex represents a small rearrangement in the white region is discussed in the light of the appearance of the unexpected crossover class of white phenotype.

Data are presented which indicate that neither Brownex nor the derived white class is due to mutation at more than one site in the white region, and furthermore, that the exceptional white class probably does not arise as the result of unequal crossing over. The possibility that the unusual white class represents a small rearrangement is considered.

The unusual spatial relationships of the mutants apricot, blood, Brownex and buff, as determined by the exceptional classes obtained from some of the heterozygous combinations, are recorded and discussed briefly.

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RELEASE OF GENETIC VARIABILITY THROUGH RECOMBINATION. II. DROSOPHILA PERSIMILIS

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RECOMBINATION between loci on chromosomes taken from a well adapted population releases an extensive array of variability (DOBZHANSKY 1946; WALLACE *et al.* 1953). To compare the amount of variability provided by recombination of linked genes affecting viability in two sibling species of Drosophila the experiments described here on *Drosophila persimilis* recombination follow the experiments in the preceding paper by SPASSKY *et al.* (1958) in basic plan.

D. persimilis is much less widely distributed than *D. pseudoobscura* and is more narrowly adapted since it is found commonly only in cooler and more moist habitats in western U. S. A. and southwestern Canada while the latter species occupies warmer and drier areas as well throughout western North America and Central America south to Guatemala. The populations of both species are highly heterozygous containing a great variety of gene complexes most of which are disadvantageous when homozygous: they do not differ significantly in the frequency of lethal or semilethal chromosomes (33.0 ± 4.5 percent in *pseudoobscura*, 25.5 ± 4.2 percent in *persimilis*) although the mean viability of homozygotes for the second chromosome (which is controlled in these experiments) seems lower in the former (55.2 ± 3.1 percent of control) than the latter species (67.6 ± 2.9 percent of control) (DOBZHANSKY and SPASSKY 1953). On the other hand spontaneous mutation rate seems higher in *D. persimilis* by a factor of nearly two over that in *D. pseudoobscura* (DOBZHANSKY, SPASSKY and SPASSKY 1954).

EXPERIMENTAL METHODS

The plan of the experiments is the same as that described in the preceding paper (SPASSKY *et al.* 1958), namely, second chromosomes which produced "normal" or subvital effects on viability were chosen from samples of flies taken from two different localities, ten chromosomes from each locality. A total of 45 intralocality crosses from each locality and 100 interlocality crosses were made. Single F_1 females resulting from these crosses were tested for viability of recombination homozygotes by a marker technique described below. In addition the twenty balanced chromosomes were retested for their effects in homozygous condition at the termination of the experiment (approximately two and a half years after its initiation). Each of these original chromosomes was tested in six replicate cultures as in the preceding reference.

[1] The work reported here was done under Contract No. AT-(30-1)-1775, U. S. Atomic Energy Commission.

Wild *D. persimilis* were obtained by the author in the summer of 1953 from two localities in the Yosemite region of California. One locality, called "South Fork", is in a dense fir forest at an elevation of 7000 ft. near the south fork of the Tuolumne River (and about 12 miles east of Mather, California); the other locality, called "White Wolf", is seven miles to the east of South Fork at an elevation of 8000 ft. in sparse lodgepole pine woods adjacent to a small pond not far from White Wolf Lodge. These localities are not far from each other but they are ecologically diverse.

The following year male descendants of these flies from 15 SF (South Fork) stocks and from 15 WW (White Wolf) stocks were mated to females homozygous for the recessive mutant cardinal (*cd*) on chromosome II. F_1 males from each cross were mated in single pairs to females in which one second chromosome contains the mutant cardinal, the dominant Delta, and an extensive inversion. The following generation Delta non-cardinal flies were inbred brother to sister. Since Delta is lethal in homozygous condition the progeny should consist of 33.3 percent non-Delta to 66.7 percent Delta if the wild chromosome contains "normal" viability factors. (See DOBZHANSKY and SPASSKY 1953, for description of this method).

From the SF and WW matings ten strains which produced closest to "normal" viability were chosen for the recombination experiment. They were kept in the balanced condition and maintained at 16°C, transferred each generation in the following way: Single pair and mass-mated (10–20 pairs) cultures were kept for each strain; the mass matings were reserved for insurance against loss and consisted of brothers and sisters from the single pair matings.

Intercrosses between strains were made and single F_1 females were mated to *cd/cd* males. Ten F_2 sons heterozygous for *cd/* interstrain chromosome II were then crossed to Delta *cd/ + cd* females. Finally F_3 brothers and sisters heterozygous for Delta/interstrain chromosome II were mated (from 2–4 pairs per culture) to yield an F_4 of which 100 flies were counted from each culture. For each interstrain cross therefore 1000 flies were counted in F_4. These F_4 cultures were raised at 25°C.

All stocks including the tester stocks were made homokaryotic for the Whitney arrangement of chromosome III.

Viability in intrapopulational crosses

The data on the effects of recombination between chromosomes of single locality origin are given in Table 1 (South Fork) and in Table 2 (White Wolf). Both tables are constructed as in the preceding paper, namely with strain numbers on the top and left margins, Roman type above the diagonal to the right are average numbers of wild type (non-Delta) flies for the ten F_4 cultures arising from recombination between interstrains; italic type below and to the left of the diagonal give variances to be discussed below, and the diagonal gives means and variances for the six replicate cultures of each original chromosome.

In every case in SF the crossover general effect is a lowering of viability (com-

TABLE 1

Viability (Roman type above and to the right of the diagonal) and variance (italic type below and to the left of the diagonal) of the recombination products of ten original chromosomes (nos. 4, 10, 12 . . . 43) from South Fork. $\bar{M}$ *and* $\bar{V}$ *= mean viability and mean variance of recombination products, or the general effect of a given original chromosome. The squares along the diagonal show the viability (Roman) and the variance (italic) of original chromosomes*

	4	10	12	21	25	27	29	30	38	43	$\bar{M}$
4	37.2 *-6.76*	31.5	30.6	30.4	27.8	32.7	27.6	29.7	25.6	22.4	28.70
10	*13.36*	30.5 *14.60*	29.9	29.5	27.6	28.9	23.5	32.8	30.7	34.7	29.90
12	*-4.31*	*10.25*	31.0 *-2.68*	31.6	25.7	26.5	29.4	18.1	32.3	23.1	27.47
21	*1.73*	*75.70*	*4.90*	35.3 *-12.64*	31.8	20.8	29.3	32.8	31.0	34.2	30.16
25	*0.15*	*-3.50*	*17.13*	*46.53*	27.0 *21.49*	26.7	26.4	28.7	21.3	25.2	26.80
27	*0.90*	*53.35*	*-0.98*	*102.37*	*20.66*	33.7 *2.26*	31.6	24.5	27.6	27.7	27.44
29	*68.95*	*80.91*	*-5.60*	*-3.38*	*-4.10*	*17.50*	31.5 *-6.18*	31.0	27.3	11.0	26.34
30	*-9.31*	*12.13*	*253.95*	*7.25*	*42.22*	*6.67*	*-8.28*	33.0 *5.90*	30.8	31.9	28.92
38	*-4.56*	*-2.16*	*-0.53*	*-3.83*	*136.80*	*8.95*	*20.16*	*5.09*	33.3 *15.59*	25.6	28.02
43	*85.99*	*6.90*	*10.56*	*51.90*	*2.88*	*9.54*	*200.21*	*-3.18*	*88.95*	30.2 *27.12*	26.20
$\bar{V}$	16.99	27.44	31.71	31.46	28.75	24.33	40.71	34.06	27.65	50.42	

pare the original chromosome viability with the corresponding average in the right hand column). Except for two strains (WW 3 and WW 11) the same is true for WW. But the average for the controls (original chromosomes' mean SF = 32.1 percent, mean WW = 31.7 percent) is higher than the recombination averages (intracrosses SF=27.99 percent, WW=28.74 percent, intercrosses=27.75 percent). SPASSKY *et al.* (1958) found this lowering of viability except in cases where control viabilities were quite low, and they explain this phenomenon as a regression of recombinants away from the higher viability of the chosen controls

TABLE 2

Viability and variance of the recombination products of ten original chromosomes from White Wolf. For further details see Table 1 legend and text

	3	11	12	13	14	15	18	21	25	31	$\bar{M}$
3	28.3 *20.31*	31.0	32.7	32.4	32.3	30.9	30.3	28.0	27.7	28.2	30.39
11	*23.94*	28.5 *0.22*	32.2	32.4	27.7	31.8	32.4	26.9	29.9	31.0	30.59
12	*3.33*	*9.90*	32.8 *-2.24*	28.2	33.4	18.5	29.7	29.0	31.9	27.2	29.20
13	*-10.12*	*-2.08*	*-6.96*	31.0 *3.41*	28.4	28.0	31.2	27.6	30.3	27.6	29.56
14	*-13.86*	*6.87*	*-1.31*	*9.67*	35.2 *-6.21*	28.4	27.5	28.7	30.3	30.5	29.68
15	*-0.36*	*8.71*	*360.36*	*11.62*	*40.38*	32.5 *-4.94*	27.6	26.5	29.1	30.0	27.87
18	*-12.68*	*10.81*	*-10.20*	*-8.63*	*-7.44*	*5.58*	33.7 *20.66*	26.0	30.5	13.4	27.62
21	*11.17*	*27.55*	*-3.92*	*-4.82*	*36.21*	*11.69*	*-5.24*	33.8 *-11.38*	29.1	25.7	27.50
25	*10.43*	*9.36*	*-2.74*	*3.55*	*1.34*	*37.93*	*-4.92*	*10.80*	32.7 *-19.01*	21.0	28.87
31	*6.86*	*-5.17*	*-5.58*	*0.69*	*6.19*	*9.90*	*189.33*	*52.69*	*129.18*	28.2 *-14.85*	26.06
$\bar{V}$	2.08	9.99	38.06	-0.79	8.67	53.98	17.40	15.11	21.66	42.68	

towards the mean viability of chromosome homozygotes in the wild population from which they were taken. These data from *D. persimilis* parallel those from *D. pseudoobscura* but show a slightly smaller effect: the average lowering of recombinants with respect to their controls (intralocalities together) in *persimilis* amounts to 3.52 percent, but is 7.38 percent for *pseudoobscura* from Mather and 3.55 percent from Texas.

Random samples of chromosome II homozygotes obtained from Mather 1951 populations of *pseudoobscura* and *persimilis* give overall viabilities of 19.82 percent and 23.32 percent respectively with more than 100 chromosomes tested for each (DOBZHANSKY and SPASSKY 1953). In the 30 strains of *persimilis* tested in preparation for these experiments before choosing 20 normals, the overall average

for SF was 19.0 percent and for WW 25.7 percent, or 22.35 percent for the two localities together, which agrees well with the earlier large sample of DOBZHANSKY's from Mather.

Viability in interpopulational crosses

The data given in Table 3 are the averages of ten homozygote viabilities per strain cross for recombination between SF and WW chromosomes. Means ($\bar{M}$) for all tests in which a chromosome is involved are given along the bottom and right margins.

The grand mean of 27.75 percent is very close to those means of the intrapopulational crosses, namely, 27.99 percent for SF and 28.74 percent for WW. It has

TABLE 3

Viability of recombination products of chromosomes derived from South Fork and White Wolf
$\bar{M}$ = the mean viability of the recombination products from crosses in which a given original chromosome participated

		S	O	U	T	H		F	O	R	K
	4	10	12	21	25	27	29	30	38	43	$\bar{M}$
WOLF	3	28.9	32.4	32.5	32.5	22.9	32.1	29.2	26.9	27.0	31.1	29.55
	11	31.0	33.5	30.3	31.8	22.1	30.9	35.6	27.2	26.0	24.4	29.28
	12	28.3	24.3	25.0	31.7	31.2	32.7	33.0	23.5	33.1	26.0	28.88
	13	32.1	15.2	24.1	30.7	23.6	15.9	29.4	10.8	30.7	25.9	23.84
	14	27.1	31.9	24.8	31.8	27.5	31.1	30.5	30.6	29.0	22.7	28.70
WHITE	15	30.0	26.3	25.1	32.8	23.5	23.8	27.6	31.1	30.2	26.2	27.66
	18	29.9	32.9	27.8	29.1	26.7	30.1	30.0	29.8	29.2	20.0	28.55
	21	29.0	28.1	27.2	26.7	25.7	31.0	36.5	27.8	29.4	29.4	29.08
	25	26.1	29.3	28.7	29.8	12.2	30.2	32.5	34.4	21.4	20.4	26.50
	31	24.8	30.3	26.8	28.8	28.4	21.8	25.5	32.2	25.8	10.4	25.48
	$\bar{M}$	28.72	28.42	27.23	30.57	24.38	27.96	30.98	27.43	28.18	23.65	27.75

been demonstrated (SPIESS 1950, and unpublished data) that populations of *D. persimilis* occupying the Yosemite transect may differ markedly in their adaptive genotypes on chromosome III though populations may have come from localities only 30 miles apart. It is then to be expected that even this short distance separating SF and WW and the diversity of ecology in those areas allows for some genetic divergence between local populations. Nevertheless recombination products of chromosomes from the same or from different populations are not significantly different in viability, a fact which agrees completely with the situation in *D. pseudoobscura*.

It is also interesting that in all cases except strains WW 3 and WW 11 the interlocality recombination average effects were lower than their original chromosome viabilities. These two strains displayed slight rise in their average effects in the intralocality effects as well. Of course both are relatively low in the control in comparison with other WW strains. In other words, average effects that are displayed in intra- and interlocality recombination hold true for particular strains but not for others. This will be discussed in more detail below in the analysis of variance.

Variance in the intrapopulational crosses

Variances given in Tables 1 and 2 in italics have been computed for each group of ten cultures with recombination chromosomes and for each group of six cultures with original chromosomes. As in the preceding paper the crude mean square (U) is obtained as the sum of squared deviations from the mean of each group divided by nine or by five, as the case may be, and is decreased by the binomial sampling error to give a residual variance (V) which represents that contributed by environmental plus genetic differences between cultures.

In SF the average residual variance for each chromosome's general effect on recombination is greater than the corresponding variance for replicate cultures of original chromosomes. The grand mean variance is 31.35 for recombination chromosomes while control variance average is 5.87.

In WW the grand mean variance (20.89) is greater than the control mean variance (−1.40) also and by nearly the same amount, but there are three chromosomes here which do not show any detectable increase in variance after recombination (WW3, 13, and 18).

Variance in the interpopulational crosses

The residual variances due to genetic and environmental differences between interpopulational recombination are given in Table 4. The grand mean of 28.36 falls between the two variance values of intrapopulational crosses. All the mean variances ($\overline{V}$) are higher than their controls including the three WW chromosomes mentioned above which produced less variance in their intralocality recombination. Especially noteworthy is WW13 which had produced no positive genetic-environmental variance when crossed to other WW chromosomes, but produces the highest variance of any WW chromosome, when crossed to SF

chromosomes. Such an effect emphasizes the nonpredictability of a recombination product based on one set of data. A chromosome in recombination with one population produces an effect entirely different from what it produced with a second population. In contrast there are quite a few chromosomes that are consistently variance producers or uniformity producers between these experiments: SF43, 25, 30, and WW31 seem to produce relatively high variances consistently while WW18, 14, and 21 consistently produce relatively low variances both intra- and interlocally.

There is then no significant difference in the amount of variance released by intralocal chromosomes in contrast with interlocal chromosomes.

TABLE 4

Variance (V) *of the viabilities of recombination products of chromosomes from South Fork and White Wolf.* $\bar{V}$ = *mean variance within crosses involving a given original chromosome*

		S	O	U	T	H		F	O	R	K
	4	10	12	21	25	27	29	30	38	43	$\bar{V}$
WOLF 3	11.77	4.14	26.12	14.78	169.33	-16.14	-0.05	-0.89	38.29	0.89	24.82
WOLF 11	41.28	9.05	0.45	-2.80	99.32	24.53	122.18	-10.18	-1.46	4.71	28.71
WOLF 12	24.59	-7.96	5.47	-2.53	-7.69	-12.01	3.67	3.13	13.52	127.87	14.81
WOLF 13	64.08	169.78	41.38	15.61	11.53	70.29	-8.32	128.32	8.95	77.48	57.91
14	1.19	-1.16	6.64	9.15	22.78	6.67	-1.37	101.03	-0.15	13.57	15.83
WHITE 15	16.78	-0.94	23.97	25.03	88.24	18.70	1.58	17.34	4.92	60.66	25.63
WHITE 18	-3.30	4.46	72.77	20.80	0.44	5.73	14.11	17.92	-0.94	51.11	18.31
WHITE 21	48.97	18.57	-4.73	31.76	18.90	-10.28	18.65	-1.89	17.68	39.02	17.66
WHITE 25	27.48	8.85	33.77	18.15	243.91	-13.35	-6.11	-9.86	6.56	63.80	37.32
WHITE 31	-6.65	7.77	13.94	45.45	61.94	8.02	55.06	17.46	77.53	145.84	42.64
$\bar{V}$	22.62	21.26	21.98	17.54	70.87	8.22	19.94	26.24	16.49	58.49	28.36

Release of variability

The magnitude of "real" variance in *persimilis* is of considerable interest for comparison with that observed in *pseudoobscura*: SF control chromosomes give a crude variance ($\overline{U}$) between replicate cultures of 27.56. Their mean viability (32.10 per cent) gives a binomial sampling variance of 21.80, the difference of 5.76 being a measure of "real" variance for SF controls. The average real variance ($\overline{V}$) for the ten control chromosomes is 5.87 ± 4.21, which is the figure preferably used in later analysis. The original WW chromosomes gave the values: $\overline{U} = 20.16$, mean viability $= 31.67$, binomial variance $= 21.64$, and $\overline{V} = -1.40 \pm 4.23$. The $\overline{V}$ values for controls are not significantly different from each other or from zero.

On the other hand recombination produces an average real variance in each experiment which is significantly greater than the control variances, namely South Fork intra $\overline{V} = 31.35 \pm 8.3$, White Wolf intra $\overline{V} = 20.89 \pm 6.5$, and interlocality $\overline{V} = 28.36 \pm 5.3$. While this increase in variance over the controls is significant, there seems to be no significant difference between the three values. Nevertheless in comparison with values obtained by SPASSKY *et al.* (1958) for *pseudoobscura* variances *persimilis* produces significantly less: the former shows an increase in variance of about 16 times its control average variance, while the latter shows an increase of only six times its control average variance.

In terms of standard deviation and the range of viabilities obtainable, *persimilis* original chromosomes have an average σ of approximately two and a grand mean viability of 31.8. In contrast recombinant chromosomes have a standard deviation close to five, a grand mean viability of 28.1, so that most recombinants are likely to range from semilethal (about 18) to supervital (about 38), or a range about two to three times that of chromosomes before recombination. The distribution of viabilities for chromosomes tested is however bimodal, a fairly symmetrical distribution around a mode of approximately 29.0 and a second small mode just about zero (lethals and extreme semilethals). The increment in range, then, is not so great and the probability of producing extreme phenotypes such as a lethal by recombination is much less than in *pseudoobscura*.

Analysis of variance for mean viability

Computation and tests of significance for variances are parallel with those in the accompanying papers. The results of the analysis of variance are summarized in Tables 5 and 6. Statistical methods are given in LEVENE (1959).

The total variability of viability is separated into genetic and residual components. The former is further subdivided into genetic variability between crosses (between average effects of recombination arising from particular pairs of chromosomes) and genetic variability within crosses (magnitude of variance between ten recombinants from any pair of chromosomes). The remaining variability is subdivided into environmental variance and residual genotype variance (which can be estimated by control), and binomial sampling variance.

The genetic component can be further subdivided into that produced by dif-

TABLE 5

Analyses of variance

Description	On means			On variances	
	d.f.	F	P (%)	F	P (%)
		South Fork intrapopulational cross			
Main effect	9, 35	0.96	40 –50	0.63	75
Interaction	35,405	4.04	⩽0.1	6.23	⩽0.1
Int. of int.	9, 35	1.06	25 –50	1.63	10 –25
Within	405, 50	1.85	<0.1	3.86	⩽0.1
		White Wolf intrapopulational cross			
Main effect	9, 35	1.79	10 –25	2.67	1 – 2.5
Interaction	35,405	2.83	<0.1	10.83	⩽0.1
Int. of int.	9, 35	4.05	0.1– 0.5	1.54	10 –25
Within	405, 50	2.04	⩽0.1	4.69	⩽0.1
		Interpopulation cross			
SF main	9, 81	2.89	0.1– 1.0	2.44	1 – 2.5
WW main	9, 81	1.92	5 –10	1.25	25 –50
Interaction	81,900	3.87	⩽0.1	3.55	⩽0.1
SF int. of int.	9, 81	2.55	1 – 2.5	3.37	0.1– 0.5
WW int. of int.	9, 81	2.17	2.5– 5	2.77	0.5– 1
Within	900,100	2.02	⩽0.1	4.67	⩽0.1

ferences between average effects which are additive and those which are nonadditive, that is acting in "epistatic," or nonadditive, ways upon recombination averages.

In no case (except possibly the SF intercross) are the additive (main) effects significantly greater than zero, while in *pseudoobscura* the main effects, though slight, are sufficient to be significant. Nonadditive effects (interactions) are all significant in *persimilis*, on the other hand, and are of about the same magnitude as in *pseudoobscura*.

SPASSKY *et al.* (1958) point out that even though additive effects are significant for *pseudoobscura* (that is, that "good chromosomes give good recombination products"), correlations between values for chromosomes under control, intra- and interconditions show little significance except that "chromosomes which give good recombination products in intrapopulational crosses tend to do so in interpopulational crosses also." From Table 7 it can be seen that these correlations in *persimilis* are not significant (with the possible exception of the SF original—SF intercross correlation) a fact which underlines the unpredictability of the recombinational effect.

The within crosses component ($\overline{V}$ minus [residual genotype + environmental effects], or $\overline{V}$ minus the control variance) is very highly significant and is larger (but not always significantly larger) than that between crosses, but as mentioned above, it is about one half to one third that found in *pseudoobscura*. In other words, chromosome recombination products are varied but less so in *persimilis*.

TABLE 6

Components of variance for means, M, *and real variances,* V. *Bold face figures are estimates. Figures above and below them are approximate upper and lower 95 per cent confidence limits respectively. Values of within crosses and residual components for* V *are for comparison only (see text). All values have two significant figures*

	Description	Additive between crosses	Non-additive between crosses	Total between crosses	Within crosses	Total genetic	"Real" residual	Binomial sampling	Total residual
MEANS (*M*)	Intra	5.7	30	..	35	..	21	21	41
	SF	**0**	**16**	**16**	**25.0**	**41**	**5.9**	**20**	**26**
		0	8.3	..	11	..	0	19	19
	Intra	7.3	16	..	30	..	9.6	21	30
	WW	**1.2**	**7.6**	**8.8**	**22.2**	**31**	**0**	**20**	**20**
		0	3.5	..	12	..	0	20	20
		16	..	..	..	..	..	..	..
	SF	**3.5**	..	..	..	..	..	..	..
		0.58	21	..	32	..	12	21	32
	Inter	———	**14**	**19**	**30**	**49**	**2.2**	**20**	**22**
		10	9.2	..	18	..	0	20	20
	WW	**1.7**	..	..	..	..	..	..	..
		0	..	..	..	..	..	..	..
	Orig	..	..	..	..	..	21	23	44
	SF	**3.7**	..	**3.7**	..	**3.7**	**5.9**	**22**	**28**
		..	..	..	..	..	0	21	21
	Orig	..	..	..	..	..	9.6	22	32
	WW	**3.1**	..	**3.1**	..	**3.1**	**0**	**22**	**21**
		..	..	..	..	..	0	21	21
VARIANCES (*V*)		0	56	..	6.2	..	..	..	3.7
	Intra	**0**	**30**	**30**	**4.3**	**34**	..	..	**1.5**
	SF	0	18	..	3.0	..	..	..	.80
		9.1	65	..	4.2	..	..	..	2.0
	Intra	**0**	**37**	**37**	**3.0**	**40**	..	..	**0.89**
	WW	0	23	..	2.1	..	..	..	.89
		11	..	..	..	..	..	..	..
	SF	**2.05**	..	..	..	..	..	..	..
		0	20	..	5.2	..	..	..	2.3
	Inter	———	**13**	**15**	**4.1**	**19**	..	..	**1.1**
		4.6	8.6	..	3.3	..	..	..	.89
	WW	**0.094**	..	..	..	..	..	..	..
		0	..	..	..	..	..	..	..
	Orig	**0**	..	**0**	..	**0**	..	..	4.3
	SF	..	..	..	..	..	..	..	**3.0**
		..	..	..	..	..	..	..	.98
	Orig	**0.16**	..	**0.16**	..	**0.16**	..	..	2.3
	WW	..	..	..	..	..	..	..	**1.6**
		..	..	..	..	..	..	..	.98

TABLE 7

Correlation between the means in different experiments for South Fork (above the diagonal) and White Wolf (below the diagonal) chromosomes

	Original chromosomes	Intra-populational	Inter-populational
Original chromosomes		.50	.62
Intra-populational	—.14		.47
Inter-populational	.17	.31	

Analysis of variance for variances

As far as the components of variance for the real variances (V) are concerned (lower half of Table 6) the additive (main) component is again nonsignificant except for SF interpopulational crosses. On the other hand the nonadditive component in all three experiments is highly significant. It can be seen that the between crosses components for variance are all much higher than the corresponding theoretical within crosses components, due to the magnitude of this interaction component in every case. In *pseudoobscura* the nonadditive components were not consistently significant and the total between crosses variance component nearly equalled the within crosses theoretical variance component. In other words while the amount of variability released is not so great in *persimilis* as in *pseudoobscura* the amount that is observed can be largely attributed to real differences between chromosomes in determination of interaction effects.

The additive F values for variances of Table 5 were calculated by nonparametric methods, while the components given in Table 6 were calculated in the usual way. In general, agreement in nonsignificance between these two holds, except for the WW main effect F (2.67) which is significant yet that component in Table 6 is not significantly greater than zero. Apparently there is a significant component but its magnitude cannot be estimated by the usual methods, yet it probably is not very far from zero in any case.

In Table 5 the interaction of interactions, or a measure of differences in the size of nonadditive effects for different chromosomes, or the predictability of the results for different chromosomes, is significant for viabilities WW intra- and both SF and WW intercrosses; and in both cases the nonadditive effects are significant. They are also significant for intercross variances.

It is useful to make comparisons between inter- and intrapopulational cross components of variance in viability: There is a slightly smaller genetic component for WW intra- than either SF or the interpopulational crosses; and this drop is chiefly due to a smaller interaction (nonadditive) component in WW (7.6). WW chromosomes when recombined with other chromosomes from the same population produce slightly fewer differences in nonadditive viability although their average ability to produce recombination effects within crosses is not significantly less than other intra- or interpopulational chromosomes. It is certainly evident that no marked change occurs in the magnitude of recombina-

tional viabilities when chromosomes from different populations are crossed in contrast with crosses of chromosomes from the same population.

In the components of variance for variances however there seems to be a slightly significant greater lowering of variance between crosses of chromosomes from different populations than between crosses from the same population (values of 15 *vs.* 30 and 37 respectively). The theoretical within crosses components are very close so that consequently the total genetic component for interpopulational variance remains less than for intrapopulational variances. In other words the genetic variances which come about through specific recombinations of particular chromosome pairs are slightly more different when the chromosomes come from one population than when they come from two different populations. SPASSKY *et al.* (1958), however, found that there were no significant differences either for the means of *M* and *V* or the components of variance of *M* and *V* in *pseudoobscura*.

Whether this minor difference between the results of *persimilis* and *pseudoobscura* recombinational effects is of fundamental importance, it is premature to say. If it is important the proximity of localities from which flies were taken in *persimilis* might be a possible clue, though by no means an explanation of this difference.

Lethal chromosomes

From inspection of Table 8 it can be seen that 19 out of 900 "intrarecombination" chromosomes and 26 out of 1000 "interrecombination" chromosomes were lethal in homozygous condition (or 2.1 percent and 2.6 percent respectively).

TABLE 8

Numbers of lethal chromosomes obtained in different crosses together with mean viabilities and variances in cultures when the presence of lethals is disregarded

Cross	Lethals	Mean viability	Variance (residual)
SF4 × SF43	1	24.9	27.92
SF12 × SF30	4	30.2	25.90
SF29 × SF43	5	22.0	152.84
SF38 × SF43	1	28.4	9.17
WW12 × WW15	4	30.8	198.09
WW18 × WW31	3	19.1	157.71
WW25 × WW31	1	23.3	84.88
SF10 × WW13	4	25.3	1.76
SF25 × WW 3	2	28.6	32.72
SF25 × WW25	6	30.5	−1.53
SF27 × WW13	5	31.8	34.51
SF30 × WW13	5	21.6	1.3
SF38 × WW31	1	28.7	−4.21
SF43 × WW12	1	28.9	51.08
SF43 × WW13	1	28.8	−5.01
SF43 × WW31	1	23.7	49.56

The means and variances of nonlethal cultures remaining out of ten cultures counted are also given in that table and can be compared with those in Tables 1–4. Only seven intrapopulational and nine interpopulational crosses produced at least one lethal (or 8.4 percent of the 190 crosses made).

It might be emphasized at this point that all control counts were made towards the end of the experiment. In one strain (WW3) which had been nearly finished at the time, the entire control count showed lethality and the strain had to be reextracted from the wild strain, tested, and crossed out again for recombination to all other strains. Undoubtedly such a change to lethality was due to point mutation, since the chromosomes were maintained in the balanced condition. On the other hand, since SPASSKY *et al.* (1958) have shown that it is likely that recombination between nonlethal complexes may engender homozygous lethal compounds in *pseudoobscura*, or synthetic lethals, it is important to weigh the evidence in *persimilis* for the likely origin of these lethal homozygotes.

It has been shown that even if all interstrain F_1 heterozygous females carried a recessive lethal, the maximum number of crosses giving one lethal culture out of ten cultures counted would be two crosses out of 190. Since there were seven such crosses it is more likely that if the lethality is due to point mutation it must have occurred subsequently, namely in the F_2 males tested in those seven crosses. On the other hand there are one cross with two lethal cultures, one cross with three lethal cultures, three with four, three with five and one with six or a total of nine crosses which produced nearer expected 0.5 probability of sons carrying the lethal from a heterozygous mother. This distribution can be compared with an expected distribution among nine samples of ten each with $p=\frac{1}{2}$ (disregarding the samples with one or no lethals). A chi-square test gives $x^2=2.44$ with $p=80$ percent. These can therefore be accounted for on the distribution following heterozygosity of the strain cross F_1 mother.

The other seven single lethal crosses are more easily explained by mutation in the F_2 sons tested. Out of the total number of sons tested how many are likely to have point lethals produced? Mutation rate on chromosome II in *D. persimilis* was measured by DOBZHANSKY *et al.* (1954) at room temperature to be 1.31 percent per chromosome generation. There were 1900 F_2 chromosomes tested in which mutation at that rate was likely to produce an average of 25 lethals appearing as one lethal containing culture out of ten. Perhaps one contributory factor to the low number of observed lethals might be the fact that numerous F_1 and F_2 crosses were made at 16°C, and lowered temperature slows the mutation rate.

Lethality among the recombination chromosomes of *persimilis*, then, is not of any magnitude nor is it distributed in any way which cannot be explained by point mutation. From the general discussion of range (see "Release of variability" above) of viability it is not expected that many extreme phenotypes would be engendered by recombination in this species.

Nonlethal cultures

If all observed lethal chromosomes (2.4 percent of the total) were produced by point mutation, the main changes in viability and variance would still be more or less the same. If these lethal chromosomes are disregarded in computation of means and variances, it can be seen that the general averages show little change. The new values would be as follows:

	Mean Viability ($\bar{M}$)	*Variance* ($\bar{V}$)	
SF intralocaltiy	28.63%	22.17±	5.58
WW intralocality	29.19%	15.60±	6.00
Interlocality	28.52%	17.86±	3.96

The mean viabilities have all increased less than one percent, while the variances have decreased in parallel but are still significantly greater than their controls.

A recalculation of variance analysis on these data excluding lethals has not been done since they amounted to such a low percentage of the total.

Recombination effects in two sibling species

The more narrowly distributed and adapted species, *D. persimilis*, consistently displays less variability after recombination than its related species, *D. pseudoobscura*. To what phenomena can this contrast be ascribed?

Before discussing intrinsic differences between these species, we should look for contrasts between the experiments reported herein and in the paper of Spassky *et al.* (1958). The *persimilis* control chromosomes chosen were higher in viability than those in *pseudoobscura*. Very often those strains which showed lowest original viability gave greatest variance in *persimilis;* though that correlation was not tested for significance, it may be that if slightly lower viability chromosomes were chosen in *persimilis* a greater similarity with *pseudoobscura* might result.

According to Dobzhansky (1946, 1955) and Wallace *et al.* (1953) who discuss the balance theory of fitness which depends on high heterozygosity in a population, such heterozygosity must be maintained by providing a variety of alleles at each locus on structurally homozygous chromosomes. Alleles preserved by natural selection must give high fitness not only by allelic interaction (heterosis) but by nonallelic interaction, or compatibility between heterozygous loci as well. If a small sample of such chromosomes is allowed to recombine and homozygotes measured phenotypically, it may be reasonable to infer a relative estimate of heterozygosity from the magnitude of variability obtained. However, a measure of heterozygosity can be obtained simply by getting the frequency of recessives in a population. California populations of *pseudoobscura* and *persimilis* did not differ significantly in the frequency of lethals and semilethals (although the latter's frequency was slightly lower than the former's). Rather the interpretation of less variability produced by recombination in *persimilis* may mean less epistatic interaction of nonallelic loci, though heterozygosity may be equal to that

in *pseudoobscura*, that is, recombination's effect is less drastic and produces less extreme viabilities. If that is so, *persimilis* either has not yet evolved epistatic systems as great as *pseudoobscura* or they are not as adaptively valuable to *persimilis* in the area sampled.

It is important to remember that the epistatic effects on viability are selected for their superiority when most loci are heterozygous. The proper balance between heterozygosity and epistatic effects between loci must be maintained. If *persimilis* can maintain heterozygosity equal to *pseudoobscura* without evolving equal epistatic interaction, either a higher mutation rate must exist in that species or recombination must be suppressed to preserve heterozygous blocks. The mutation rate *is* higher in *persimilis*; from the lack of structural heterozygosity on chromosome II in these species we may assume equal crossing over for them. On the other hand, if *persimilis* is really less heterozygous than *pseudoobscura*, less change would be expected from recombination: if more homozygosis exists, natural selection should eliminate deleterious recessives *and* bad epistatic interactions in homozygous recombinants. Consequently from the observation that *persimilis* shows a less drastic recombination effect, a proportional decrease in heterozygosity is not necessarily to be inferred.

SUMMARY

Two samples of ten second chromosomes each of *Drosophila persimilis* were chosen for their near normal viability in homozygous condition from populations occupying localities in Yosemite National Park, California.

A total of 1900 recombinant chromosomes from these original 20 were tested for viability effects in homozygous condition by an experimental plan exactly parallel with the preceding and the following papers.

Recombinant viabilities are lower on the average than control viabilities, that is they regress toward the average viability of homozygotes in the populations from which they come. The range of viability observed among recombinants extended from lethality to supervitality, but neither the number nor distribution of lethals is greater than or unaccountable by point mutation. This fact is in marked contrast with *D. pseudoobscura* which certainly produced some lethals "synthetically", that is by recombination.

Analysis of variance shows a significant increase in the genetic components of variability especially in interaction (nonadditive) effects on viability and variance differences over their controls. None the less, the magnitude of this array of variance ($6 \times$ the controls) is not as great as in the sibling species, *pseudoobscura* ($16 \times$ the controls).

While it is known that both species are highly heterozygous, it is possible that the difference between them in these recombinant effects may be due to less epistatic interaction of nonallelic loci in *persimilis* than in *pseudoobscura*. On the other hand a real difference in amount of heterozygosity between these species may account for their differences in recombination effects.

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EXPERIMENTAL TRANSFER OF MATERNALLY INHERITED ABNORMAL SEX-RATIO IN DROSOPHILA WILLISTONI

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In previous communications (MALOGOLOWKIN and POULSON 1957; MALOGOLOWKIN 1958) a cytoplasmically inherited sex-ratio condition has been described in *Drosophila willistoni* and in *D. paulistorum*. In both species, certain females produce progenies consisting mostly or only of daughters. This capacity for the production of unisexual broods is inherited by their entire female progenies and is not transmitted at all by the few exceptional male descendants that appear in some cultures. Many of the eggs deposited by sex-ratio females of *D. willistoni* are recognizably abnormal within two to four hours of oviposition; and there are reasons to suppose that these dying eggs represent the male zygotes, i.e. eggs fertilized by Y-bearing spermatozoa (MALOGOLOWKIN and POULSON 1957). Although the sex-ratio condition is due to the nonchromosomal transmission of a causative agent, through the cytoplasm of the egg, it is not wholly independent of the influence of chromosomal genes. Crossing of sex-ratio females to males from strains of certain geographic origins results, after a few generations, in an irreversible loss of the sex-ratio condition (MALOGOLOWKIN 1958). These strains evidently contain genes which are less favorable for the multiplication of the causative agent in the cytoplasm than are the genes of many other strains.

The present article reports in detail the results of experiments which demonstrate that the causative agent of the sex-ratio condition may be mechanically introduced into the germinal materials of females of previously normal strains. Thus a female of *D. willistoni* may deposit the sex-ratio type of abnormal, inviable eggs within about ten days after the injection into her abdomen of ooplasm from an abnormal egg of a sex-ratio female. Moreover, at least a portion of the female progenies of such females carry the causative agent of the sex-ratio condition in their eggs, and the condition is inherited through the female lines from there on with same persistency and regularity as in the original sex-ratio lines of females.

MATERIALS AND METHODS

The sex-ratio, or unisexual, strains used in the present investigation are those reported on by MALOGOLOWKIN and POULSON (1957) and genetically analyzed by MALOGOLOWKIN (1958). They are derived from a fly collection at Bath, Jamaica, by DRS. W. B. HEED and M. WASSERMAN of the University of Texas.

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This fly, a female, was observed by MR. B. SPASSKY to yield an unisexual progeny. The resulting strain was back crossed repeatedly to a laboratory strain containing the sex-linked mutants eosin (w^e), singed (*sn*), yellow (*y*), Incomplete (*Inc*), and roughoid (*ro*), and their daughters in turn repeatedly back crossed to males of this strain. Except for a few males which appeared in the tenth generation of this backcrossing, the strain produced exclusively females. Other series of crosses and backcrosses were made to the autosomal recessive mutant pink (*p*) and to a wild strain derived from flies collected on the island of Barbados. Aside from a very few occasional males these progenies consisted of females only. These strains served as sources of eggs containing the sex-ratio causative agent.

A study was made of the proportion of the eggs of sex-ratio and of normal strains which survive and give rise to adults. For this purpose three pairs of flies from a given strain were introduced into sterilized vials and given paper spoons with fresh Drosophila nutrient medium. Ovipositing females, about three days old, of the sex-ratio strains were used; and for controls, females of similar age from various strains of diverse geographic origins giving normal progenies. The flies were allowed to oviposit for about 12 hours after which the spoons with eggs were replaced by fresh spoons. The eggs on the spoons were examined every three to six hours and the larvae that hatched were transferred to creamers containing culture medium. Counts of eggs, pupae, and adult flies were recorded.

For the injection experiments eggs were collected on spoons which were changed every four hours. The eggs were transferred into watch glasses containing WADDINGTON's modified Drosophila Ringer's solution. The eggs were washed in this with the aid of a pipette to clear them of adherent bits of yeast and medium. Most of the solution was then pipetted away and a few drops of a diluted Chlorox solution (equal parts Chlorox and Ringer's solution) added to dechorionate the eggs. This action was watched under a binocular microscope; and as soon as the chorions became transparent Ringer's solution was added and the eggs were washed repeatedly and thoroughly to remove all traces of hypochlorite.

As previously reported (MALOGOLOWKIN and POULSON 1957) the abnormal eggs are characterized by increasing translucent areas at the time of blastoderm formation and so are readily distinguishable from normally developing zygotes and from unfertilized eggs. The microinjection apparatus used was that of RIZKI (1953) which is most convenient and allows for rapid changing of micropipettes without danger of contamination. Abnormal eggs (three to six hours after oviposition) were punctured with a micropipette and the ooplasm sucked out. The ooplasm was then mixed with a small amount of the Ringer solution in the watch glass and a portion taken back into the pipette for injection into the host female. The recipient females were kept lightly etherized. They consisted of one to two day old virgin females of the Recife-3 strain of *D. willistoni* which has always yielded normal sex-ratios. The suspension of ooplasm was injected into the abdomen at about the level of the ovaries taking care not to pierce the gut. In each

instance about one third the content of an egg was injected. While an effort was made to keep the quantities as uniform as possible, there was obvious variability in some instances which were noted in the records.

The injected females were left for several hours to recover in separate sterile vials, provided each with two males of the Recife-3 strain, and fed with honey-yeast mixture on spoons of Drosophila medium. The spoons were replaced every other day, the bit of food with the two day collection of eggs being transferred into a creamer containing fresh medium. This procedure was continued throughout the life of each female so that her entire progeny was obtained as a series of successive two-day broods. The creamers were kept under close observation and in some instances unhatched eggs were removed for examination. The numbers of females and males hatching on successive days in each of the creamers was recorded.

Control experiments were carried through in precisely the same manner, except that ooplasm from unfertilized eggs laid by virgin females of the normal Recife-3 strain was utilized to inject other young virgin Recife-3 females. The injected controls all survived and all laid eggs.

All experiments were carried out at 25°C and with humidity never less than 50 percent in the incubator.

Mortality among eggs of sex-ratio females

To establish whether male zygotes are usually not formed, or die at some stage of development, it was necessary to examine mortality in normal and sex-ratio strains. Table I is a summary of certain data on the survival of eggs from several

TABLE 1

The survival of eggs in "sex-ratio" and in normal strains

Strain	Total eggs	Eggs hatched	Percent eggs hatched	Pupae	Adult		Percent total survival
					Females	Males	
Control	1,141	987	86.5	962	431	359	69.2
Sex-ratio	1,487	418	28.1	349	332	3	22.5

normal and sex-ratio strains of *D. willistoni* obtained early in the course of this study. It is seen that a total of 1141 eggs from several normal strains yielded 790 adult flies with a slight but significant preponderance of females. The percentage of eggs giving rise to adults under the conditions of experiment (which may not have been the most ideal for *D. willistoni*) was 69.2. However, the hatchability of eggs was 86.5 percent and the largest part of the mortality was clearly post-embryonic, chiefly in the pupal stage. In the case of the sex-ratio females mortality is much higher and is confined almost completely to egg and early larval stages. From 1487 eggs only 335 adults, or 22.5 percent, came through and only three of these adults were males. The hatchability of eggs (about 28 percent) is strikingly below that of any normal strains.

At the time these counts were done no attempt was made to analyze the low hatchability in developmental terms. Subsequent examination of eggs laid by sex-ratio females showed that considerable numbers of unfertilized, or unfertilizable, eggs are almost always present in the collected sample. When these eggs are taken into account the proportions of normally developing to abnormal, dying eggs is usually close to 1:1. Examination of the ovaries of mature, actively laying sex-ratio females shows that numerous large ovarian eggs are clearly aberrant and presumably incapable of being fertilized. These appear to be the source of the numercus unfertilized eggs observed among the samples collected from many sex-ratio females.

The developmental disturbance in the abnormal, dying eggs is very striking and led to the conclusion (MALOGOLOWKIN and POULSON 1957) that they represent the missing male zygotes. If so, then these eggs ought to be a potent source of the sex-ratio causative agent. Accordingly the injection experiments were planned to test this possibility.

An account of the developmental disturbances will be published separately.

Progenies of injected females

Table 2 presents the composition of the progenies of the fourteen Recife-3 females (SR) which were injected with the sex-ratio ooplasm, survived the injection, and were fertile. Table 3 presents the comparable data for the fifteen control females (UN) injected with the ooplasm of unfertilized eggs from other Recife-3 females.

It will be seen at once that the control females produced both daughters and sons as long as they remained alive and fertile. Even among the last eggs deposited by these females the proportions of the two sexes remain close to 1:1. There is considerable range in the productivity among these females. Whether this be the result of the injection of the ooplasm or normal variability cannot be told in the absence of comparable data for an uninjected series of Recife-3 females. There is no significant deviation from the normal sex-ratio here.

A very different situation is observed in some of the progenies of the females injected with ooplasm from sex-ratio eggs. For some days following the injections, females, SR-1, SR-103, and SR-109 deposited eggs from which daughters and sons developed with the expected frequencies of about 1:1. However, between the 13th and the 17th days after injection SR-1 produced 72 daughters and 19 sons, and after the 17th day 127 daughters and only one son. SR-103 gave 206 daughters and 201 sons from the eggs deposited up to the 13th day following injection, and 49 daughters and no sons thereafter. In the case of SR-109 there were 169 daughters and 182 sons from eggs laid up to the 15th day followed by 51 daughters and only four sons thereafter. The most striking case is that of SR-2 who from the beginning of egg laying on the third day gave in each brood a ratio of two daughters to each son for a total of 186 daughters and 75 sons through the 13th day, and thereafter to the 31st day 101 daughters and no sons.

Thus it appears that at least four out of the 14 females injected with the

TABLE 2

The progenies of the females injected with the sex-ratio ooplasm. The numbers of daughters and sons which developed from the eggs deposited on different days after the injections are shown. The zeros indicate that the mother was alive but deposited no eggs

Days	1–3		3–5		5–7		7–9		9–11		11–13		13–15		15–17		17–19		19–21		21–23		23–25		25–27		27–29		29–31	
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
SR–1	2	3	84	59	43	47	55	60	4	4	34	30	27	14	45	5	19	0	20	0	12	0	19	1	28	0	7	0	22	0
SR–2	0	0	33	15	47	15	45	17	32	15	29	16	14	0	25	0	27	0	11	0	2	0	0	0	0	0	0	0	22	0
SR–3	2	1	34	36	35	37	47	53	41	37	29	37	30	20	7	0	0	0	0	0	0	0	0	0	0	0	..	..	..	..
SR–5	10	13	74	42	46	32	27	17	23	26	23	20	2	4	0	1	0	0	..	..	..	..	..	..	..	..	..	..	..	..
SR–8	0	0	33	24	77	66	79	76	55	59	50	58	11	11	10	14	2	3	0	0	..	..	..	..	..	..	..	..	..	..
SR–9	18	13	64	69	51	37	41	34	18	17	16	24	0	0	1	0	0	0	..	..	..	..	..	..	..	..	..	..	..	..
SR–11	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	3	2	10	10	7	3	4	1	2	2
SR–12	16	20	61	32	46	43	50	40	45	52	29	53	17	20	43	52	0	0	0	0	1	0	0	1	0	0	0	0	0	0
SR–103	0	0	30	23	41	41	68	59	30	42	37	36	13	0	36	0	0	0	0	0	0	0	..	..	..	..	..	..	..	..
SR–105	0	0	43	37	56	48	69	66	23	24	33	36	0	0	0	1	0	0	..	..	..	..	..	..	..	..	..	..	..	..
SR–107	0	0	23	31	20	28	60	46	15	30	10	9	0	3	2	3	0	0	0	0	0	0	..	..	..	..	..	..	..	..
SR–108	0	0	8	7	0	0	18	21	0	0	0	0	0	0	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
SR–109	0	0	24	21	44	40	56	60	26	45	15	25	4	11	28	4	15	0	8	0	0	0	..	..	..	..	..	..	..	..
SR–111	0	0	12	10	16	30	0	0	0	0	0	0	1	2	2	3	5	5	4	2	2	0	0	0	..	..	..	..	..	..
SR–112	0	0	28	31	30	38	52	42	41	38	18	19	28	16	19	15	25	42	21	21	8	9	13	12	28	39	19	14	15	19

TABLE 3

Number of daughters and sons developed from eggs deposited by the control female. These females were injected with the ooplasm of unfertilized eggs of normal females

Days	1–3		3–5		5–7		7–9		9–11		11–13		13–15		15–17		17–19		19–21		21–23		23–25		25–27		27–29		29–31	
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
UN–1	0	1	33	36	26	35	50	40	35	27	27	35	24	24	24	33	8	7	..	..	..	..	..	..	..	..	..	..	..	..
UN–2	2	2	47	25	26	34	2	3	0	0	0	0	0	0	0	0	0	0	..	..	..	..	..	..	..	..	..	..	..	..
UN–3	8	13	20	23	59	63	60	53	34	27	39	34	21	26	22	19	32	26	4	1	0	0	4	1	..	..	..	..	..	..
UN–4	5	3	49	34	44	46	60	55	46	28	45	46	14	21	32	38	19	22	1	4	0	0	1	0	0	0	..	..	..	..
UN–5	18	17	41	26	49	50	32	39	11	14	28	33	7	7	3	0	1	1	..	..	..	..	..	..	..	..	..	..	..	..
UN–6	2	1	51	42	30	50	40	42	10	14	39	34	25	29	26	14	20	22	1	2	0	1	0	0	..	..	..	..	..	..
UN–7	11	11	34	20	30	31	41	44	26	38	43	48	7	5	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
UN–8	6	3	38	33	55	82	94	89	50	43	68	76	62	43	25	35	46	34	5	5	30	32	19	13	29	21	6	12	2	2
UN–9	0	0	56	46	16	20	25	27	37	25	6	8	9	3	1	5	1	3	..	..	..	..	..	..	..	..	..	..	..	..
UN–10	3	0	49	24	4	5	0	2	11	11	0	0	2	3	3	1	3	2	1	0	0	0	..	..	..	..	..	..	..	..
UN–11	3	5	7	5	0	0	0	0	30	20	0	0	0	0	0	1	0	0	..	..	..	..	..	..	..	..	..	..	..	..
UN–12	1	6	13	14	0	0	0	0	16	11	0	0	0	0	0	0	0	0	..	..	..	..	..	..	..	..	..	..	..	..
UN–13	21	13	27	21	65	58	77	76	27	36	49	47	32	37	33	37	28	34	30	37	2	2	19	21	3	2	..	..	..	..
UN–14	1	1	12	20	10	6	20	22	2	3	16	22	0	0	0	0	0	0	..	..	..	..	..	..	..	..	..	..	..	..
UN–15	0	0	14	11	5	8	1	1	0	0	26	17	28	19	6	8	1	2	..	..	..	..	..	..	..	..	..	..	..	..

ooplasm from sex-ratio eggs have become infected with the sex-ratio agent and, after a period of some days begin themselves to deposit sex-ratio eggs. This interpretation was immediately confirmed by examination of samples of eggs laid by these females during the later days of oviposition. Numerous eggs of the type characteristic of the original sex-ratio strain were found; some were photographed and fixed for embryological and cytological study. The latter were from eggs deposited by female SR-1 during the period from the 25th to the 27th day following injection.

Second generation progenies of the injected females

It is now logical to inquire whether the females in the progeny of the injected mothers will themselves manifest any deviation from the normal proportions of the two sexes in their offspring.

The number of females in the progeny of the injected mothers was obviously too great for them to be tested as fully as might be desired in the following generations. Tests had to be made selectively, but even so the records are far too bulky for full publication. Table 4 presents a summary of the data for the progenies of the daughters of injected females (this is the second generation, the in-

TABLE 4

Second generation progenies of normal females injected with "sex-ratio" ooplasm. The numbers of daughters tested from different broods and the members producing unisexual progenies are shown. The letters in parentheses indicate the broods which were used from the third generation tests reported in Table 5

Injected female	Days after injection	Daughter tested	Unisexual progenies	Injected female	Days after injection	Daughter tested	Unisexual progenies
SR–1	3–5	14(A)	0	SR–3	11–13	5(L)	0
SR–1	5–7	6	0	SR–3	15–17	4(M)	0
SR–1	7–9	6	0	SR–5	3–5	2	0
SR–1	11–13	7(B)	0	SR–5	7–9	11	0
SR–1	13–15	4(C)	0	SR–5	9–11	4(N)	0
SR–1	17–19	12	6(D)	SR–5	11–13	7(O)	0
SR–1	19–21	13	4	SR–9	7–9	5	0
SR–1	23–25	11	4	SR–9	15–17	1	0
SR–1	25–27	9	1	SR–12	3–5	6	0
SR–1	29–31	4	2	SR–12	7–9	3	0
SR–2	3–5	3(E)	0	SR–12	9–11	15(P)	0
SR–2	5–7	11(F)	0	SR–12	29–31	2	0
SR–2	7–9	1	0	SR–103	7–9	6(Q)	0
SR–2	11–13	9(G)	0	SR–103	11–13	14(R)	1(S)
SR–2	13–15	6(H)	0	SR–103	15–17	25(T)	21(U)
SR–2	15–17	16(I)	8(J)	SR–105	7–9	7	0
SR–2	17–19	2	2	SR–109	15–17	6(V)	0
SR–2	21–23	1	0	SR–109	17–19	5(W)	1
SR–2	27–29	2	1	SR–111	27–29	2	0
SR–3	3–5	1	0	SR–112	9–11	4	0
SR–3	9–11	2(K)	1	SR–112	13–15	10(X)	0

jected females being the parental and their daughters the first generation). The test progenies were obtained by mating single females from the respective cultures with two brothers where available, or with two males from other cultures of the same experimental series, or with males from the Recife-3 stock.

As shown in Table 2, the injected female SR-1 produced normal progenies, containing both males and females, for about 15 days from the beginning of egg laying. Table 4 shows that we tested 14 females from the brood produced between the 3rd and 5th days after injection, six females from the brood of the 5th to 7th days etc. Examination of the progenies of the 37 females tested through the 15th day showed normal proportions of daughters and sons. However, in the broods which the injected female SR-1 produced between the 17th and 31st days after injection there were 127 daughters and one son (Table 2). Of these daughters, 49 were tested (Table 4). These 49 progenies fall into two rather clearcut groups: 32 yielded essentially normal proportions of the sexes for totals of 1706 ♀ ♀ and 1406 ♂ ♂; 17 yielded unisexual progenies which totalled 1160 ♀ ♀ and 12 ♂ ♂; and only two cultures which might be doubtful yielding, respectively, 113 ♀ ♀ and 31 ♂ ♂, and 141 ♀ ♀ : 77 ♂ ♂.

The injected female SR-2 produced progenies showing a ratio of about 2♀♀:1♂ for the first 13 days following the injection and only unisexual progenies thereafter (Table 2). A total of 24 of her daughters produced before the 13th day, and 26 daughters produced thereafter were tested. Table 4 shows that the former yielded bisexual progenies, while 11 of the latter gave only daughters. Among the 16 tested females from the 15th - 17th brood, eight yielded no sons, two gave about equal numbers of females and males, and six produced twice or more times as many daughters as sons.

The injected females SR-3, SR-5, SR-9, SR-12, SR-105, SR-111, and SR-112 continued to produce apparently normal broods for as long as they remained alive and fertile (Table 2). Of a total of 91 of their daughters tested all but one yielded approximately normal proportions of the sexes (Table 4). The exceptional female, a daughter of SR-3, produced 16 daughters and no sons. The females SR-103 and SR-109 produced some, although small, unisexual broods toward the end of their reproductive lives. Table 4 shows that we were able to test 56 of their daughters and that 22 of these, all of them daughters of SR-103, yielded unisexual progenies. More precisely, the six females from the brood produced by SR-103 on the 7-9th days all gave normal progenies; of the 14 females from the brood on the 11–13th days, 11 gave normal progenies, one gave 69 ♀♀ and no ♂♂, another gave 134 ♀♀:14♂♂ and a third 92 ♀♀:9♂♂; of the 25 tested females from the brood on the 15–17th days, 21 gave few or no males and four gave ratios of 105 ♀♀:15 ♂♂, 194 ♀♀:38 ♂♂, 116 ♀♀:40 ♂♂, and 74 ♀♀:37 ♂♂ respectively.

The number of tested daughters from the control series was far fewer as it seemed more important to test for transmission in the injected series. We have records of tests of 37 females of the first generation of the control experiment (reported in Table 3) and have carried certain of these for a number of further

experiments. As expected all of these yielded progenies in which the proportions of the sexes closely approached 1:1.

Third and fourth generation progenies of the injected females

The data which have been presented show that the absence of males in the last broods produced by some of the injected females (Table 2) was not accidental. A part, but only a part, of the daughters in these unisexual broods yielded in turn unisexual, or near unisexual, progenies. The rule here is not as consistent as that for the original sex-ratio strain which says that daughters from unisexual broods always produce unisexual or near unisexual broods. In the present case some of the daughters from unisexual broods produce unisexual broods, others produce bisexual broods. Usually the females from bisexual broods of the injected females give bisexual progenies and their daughters continue to do so. There are some exceptions to this; for example, a single female from the bisexual brood produced by SR-3 between the 9th and 11th days following injection. This daughter (Table 4) produced a progeny consisting of 16 ♀♀ and no ♂♂. In most cultures there is no doubt as to whether a given progeny is unisexual or not: either no or very few sons are produced, or the ratio of the sexes approaches equality. Only a small minority of cultures show an intermediate situation and these have been included in Table 4 among the nonunisexual progenies.

It was clearly important to establish whether the proportions of the sexes became finally stabilized in further generations (third, fourth, and later) descended from the injected females. For this purpose some females were tested from the third generation broods marked by capital letters in the "Daughters Tested" columns in Table 4. The results of these tests for the third generation are presented in Table 5. The situation can be summarized as follows. The

TABLE 5

Third generation progenies of normal females injected with "sex-ratio" ooplasm. Numbers of females tested and numbers yielding unisexual progenies are shown. The letters designating the broods refer to the data reported in Table 4

Injected female	Second generation brood	Females tested	Unisexual progenies	Injected female	Second generation brood	Females tested	Unisexual progenies
SR–1	A	3	0	SR–3	M	3	0
SR–1	B	16	0	SR–5	N	15	0
SR–1	C	18	0	SR–5	0	24	0
SR–1	D	3	2	SR–12	P	27	0
SR–2	E	1	0	SR–103	Q	1	0
SR–2	F	24	0	SR–103	R	14	6
SR–2	G	46	3?	SR–103	S	1	1
SR–2	H	33	1?	SR–103	T	1	1
SR–2	I	26	1	SR–103	U	39	11
SR–2	J	30	9	SR–109	V	6	0
SR–3	K	5	4	SR–109	W	1	0
SR–3	L	32	3	SR–112	X	4	3

daughters from the normal-sexed broods (A,B,C,E,F,G,H,I,K,L,M,N,O,P,Q,R,-T,V,W, and X, see Table 4) produced in the third generation mostly, but not exclusively, broods with normal ratios of the sexes (Table 5). The exceptions, some of them marked in Table 5 by question marks, deserve careful consideration. Thus, females of the brood G derived from SR-2 produced three cultures with 13 ♀♀:2 ♂♂, 27 ♀♀:5 ♂♂, and 30 ♀♀:3 ♂♂, respectively. One female from brood H produced a culture with 41 ♀♀:4 ♂♂, and a female from brood I one with 84 ♀♀:0 ♂♂. Females from brood K produced cultures with 216 ♀♀:0 ♂♂, 247 ♀♀:0 ♂♂, and 194 ♀♀:11 ♂♂. Three of the females of brood L produced cultures consisting of 123 ♀♀:0 ♂♂, 119 ♀♀:0 ♂♂ and 205 ♀♀:0 ♂♂.

The daughters from the unisexual broods tested (D,J,S, and U in Table 4) produced in the third generation either unisexual or bisexual progenies (Table 5). In the cases of the larger numbers tested (J and U) the unisexual progenies amounted to nine out of 30 and 11 out of 39 respectively. Clearly the proportion of unisexual broods is much higher than in the majority of progenies derived from the bisexual broods, although in the case of brood X four out of five third generation progenies were unisexual.

The fourth generation, although tested on a smaller scale, seemed to show a better stabilization of the recently acquired "sex-ratio" condition. For instance, a culture of the third generation (of SR-2 provenience, a culture of brood J, Table 5) produced 61 females and no males. From among these three were crossed to males of the normal strain Recife-3. They produced a total of 469 females and no males. A strain was thus established which in each generation is maintained by crossing with males of the Recife-3 strain. At the time of writing this strain is in the fourteenth generation, counting from the injected female. While no exact counts of flies produced have been kept, it is pretty certain that the progenies have been unisexual or nearly so, for in no generation have any males (except those used as parents) been observed.

[As of March 1959, four transferred strains are still maintained with strong degrees of unisexuality (SR-2, SR-5, SR-12, and SR-103) on a Recife-3 background.]

From the brood U of SR-103 origin (see Table 5) 15 females from unisexual progenies were outcrossed to Recife-3 males. Their progenies (=fourth generation) aggregated 2507 females and no males. From another culture of brood U which yielded a third generation of 149 ♀♀:11 ♂♂ (a case of an intermediate ratio) three females were tested and yielded a fourth generation which was unisexual and totalled 465 ♀♀. From another culture with an intermediate ratio (125 ♀♀:25 ♂♂) four females were tested. These produced respectively the following progenies: 128 ♀♀:0 ♂♂, 128 ♀♀:1 ♂, 118 ♀♀:118 ♂♂, and 98 ♀♀:95 ♂♂.

Injections into adult males

It seemed desirable for several reasons to test the effects of the sex-ratio agent on adult males. Accordingly a small but adequate series of injections of ooplasm from abnormal eggs of the original strain of sex-ratio females into young males

of Recife-3 were made. The experiments were carried through with controls in precisely the same manner as those with females except that no progeny tests were made. The results which are presented in Table 6 demonstrate that the sex-ratio agent is lethal to adult as well as to embryonic male zygotes.

The conclusion is evident that the "sex-ratio" condition (=unisexual progenies) in *Drosophila willistoni* can be introduced by mechanical means into a strain of flies normally free from it, passed to further generations, and subsequently incorporated as a regular feature of the hereditary system of this strain. The normal method of transmission in the "sex-ratio" strains is through an agent which has so far proved to be limited to the female germ line and is transmitted through the egg from mother to daughters. This agent produces a cataclysmic disturbance of development in XY zygotes which is thus responsible for the appearance of unisexual progenies. Ooplasm from the degenerating XY eggs of females of the original "sex-ratio" strains provides a source of the agent which on introduction by injection into the abdomens of normal females becomes incorporated through "infection" into a germ line previously free from it.

TABLE 6

Effects of injection of "sex-ratio" ooplasm into adult males of Recife-3; and controls

Series	Ooplasmic donor	Host male	No. injected	No. surviving to:			
				24 hr.	48 hr.	72 hr.	96 hr.
Sex-ratio	3–6 hr. abnormal egg	Recife-3	11	4	1	0	..
Control	3–6 hr. unfertilized egg of Recife-3	Recife-3	10	10	10	10	10

DISCUSSION

That the infection is not 100 percent effective in all oogonia of both ovaries in all of the injected females is scarcely surprising. The remarkable thing is the comparative success of infection. The apparent inefficiency of artificial transfer compared with natural transmission in the original strains is perhaps to be expected. It may be due to any, or a combination, of several factors. In the first place, the phenotypic expression (with the possible exception of SR-2 in Table 2) of "sex-ratio" makes its appearance among the progeny of the injected females only after a latent or incubation period. This period of from 10–14 days represents about one half the maximum reproductive life of a female, and some females (SR-5, SR-9, and SR-108 in Table 2) ceased reproduction close to this time. Furthermore, although an attempt was made to control the quantity of ooplasm injected, and the site of the injection, there is no assurance that in each female the oogonia were exposed at the same time, or to the same quantity of the infective agent.

As another expression of inefficiency might be taken the fact that only a fraction of the unisexual broods of females continue to transmit the capacity for pro-

ducing such unisexual broods. In fact, a majority of females relapsed to the production in subsequent generations of normal bisexual progenies.

The extent to which the agent may be transmitted without phenotypic expression, as indicated by certain instances of females from bisexual broods (see Table 5) who subsequently produced unisexual progenies, remains to be fully examined. This is a point of considerable interest in relation to a feature of these experiments to be discussed below.

The decisive fact remains that in at least some of the lines of descent from the injected females the unisexual progenies become a trait which is inherited as faithfully, from the mother to her daughters, as it is in the original "sex-ratio" strain from which the transfers were made.

It is necessary now to discuss in some detail a feature of the above experiments which is due solely to chance. In a preliminary experiment (not presented here) we injected "sex-ratio" ooplasm into females of a number of different normal wild strains with some indications of success. Most of the females who survived more than a few days produced large numbers of eggs, a relatively small proportion of which showed any normal development, the majority resembling the degenerating abnormal or "unfertilizable" eggs commonly found among the eggs of the original "sex-ratio" strains. With this favorable indication the more extensive series of experiments reported in this paper were begun. At this time the most favorable culture for obtaining large numbers of virgin wild females proved to be one of the Recife-3 strain (derived from wild progenitors collected near the city of Recife, Brazil). Now it happens, as was demonstrated by the genetic studies of MALOGOLOWKIN (1958), that this strain is the *least favorable* for the perpetuation of the "sex-ratio" condition via the normal mode of transmission. The Recife-3 strain contains genes which disrupt the "sex-ratio" when females from a "sex-ratio" strain are repeatedly outcrossed to males of this strain. Indeed, an excellent therapeutic for the "sex-ratio" condition is to cross with Recife-3 males! Thus it is probable that much of the inefficiency and irregularity in expression and transmission which were encountered represent a direct reflection of this genotypic resistance. It is therefore likely that injection of "sex-ratio" ooplasm into females of the strains which were found (MALOGOLOWKIN 1958) to be genotypically more favorable for the perpetuation of the causative agent would result in easier "infection" and more complete transmission of the unisexual condition to the progenies of injected females. It might also lead to such high infections as not to allow for the production of any normal eggs, resulting in the laying of nothing but "unfertilizable" eggs. The preliminary experiments mentioned briefly above provide some evidence in this direction.

The possession of an unfavorable genotype by the strain utilized in the experiments strongly suggests that other factors than these which were discussed above also came into play in the course of the establishment of the unisexual strains which we were able to derive in the later generations. While the introduction to and maintenance of the "sex-ratio" agent in a genetically favorable environment may be regarded largely as a problem of physical or mechanical

factors, an equivalent introduction to and maintenance in an unfavorable, or even hostile, genetic environment certainly involves problems of adaptation as well. In our case the "sex-ratio" agent has either produced changes in the host's cytoplasmically transmitted materials, or it has itself undergone mutation and subsequent selection. Little is yet known about the ultimate nature of the agent except that it is submicroscopic, and we cannot say whether it may be a single type of particle or a mixed population in the original "sex-ratio" strain. Which of these possibilities may prove correct can scarcely be decided until further information becomes available concerning the agent and its mode of multiplication. The situation here may have a good deal in common with the cyclic nucleocytoplasmic interactions reported by SONNEBORN (1954) in Paramecium.

Available evidence indicates that in the original "sex-ratio" strain synchronization of multiplication of the agent with the other genetic materials of the host female is very close indeed. While occasional failure of the agent to become incorporated into an egg allows for development of exceptional males, the frequencies of these and of bisexual females is not influenced by temperature as shown by MALOGOLOWKIN (1958). It is quite possible that in the genotypically unfavorable strains the "sex-ratio" agent may be in strong competition with other particles normally present in the cytoplasm and in such an internal environment may be more sensitive to external agencies, such as temperature which MAGNI (1954) found to affect profoundly the "sex-ratio" condition in certain strains of *D. bifasciata* investigated by him.

It is not profitable at present to speculate extensively concerning the nature of the causative agent of "sex-ratio" in *D. willistoni*. While comparison with certain viruses suggests itself, it is more appropriate to limit our comparisons primarily to instances in Drosophila. The classical case of sensitivity to carbon dioxide discovered and studied by L'HERITIER and his collaborators (1946, 1947, 1951, 1955) provides some interesting analogies. L'HERITIER and DE SCOEUX (1947) demonstrated that the "genoid," or virus which causes the heightened sensitivity to CO_2 can be transmitted from generation via the egg (also male gametes) or, artificially by injection of hemolymph or certain other organs of sensitive strains into previously resistant ones. While the parallel with "sex-ratio" in *D. willistoni* is close in most respects there are some interesting differences e.g., CO_2 sensitivity can be transferred by injection of hemolymph and other organs, but indications are strongly that the "sex-ratio" agent is confined to the germ-line in females and is completely lethal in XY zygotes and adult males.

Another parallel, already discussed by MALOGOLOWKIN (1958) is the situation in *D. prosaltans* in which the "sex-ratio" strain discovered by CAVALCANTI and FALCÃO (1954) and analyzed by CAVALCANTI, FALCÃO and CASTRO (1957) is perpetuated, or disrupted, depending on the presence or absence of certain nuclear genes. The intriguing example of "sex-ratio" in *D. bifasciata* investigated by MAGNI (1953, 1954) while having some features in common with these differs from all of them, as mentioned above, in being profoundly affected by temperature. In turn these cases suggest resemblances to the well known "Killer"

trait in Paramecium worked out in the now classical studies of SONNEBORN (1943a, 1943b, 1951) and his collaborators (PREER 1948).

In the cases both of CO_2 sensitivity in *D. melanogaster* (L'HERITIER 1951, 1955) and the "Killer" character in *Paramecium aurelia* (DIPPEL 1950) mutant forms of the cytoplasmic particles are known and have been extensively studied. It seems not unlikely that mutant forms of the "sex-ratio" agent of *D. willistoni* may be distinguished sufficiently to employ them in the type of analysis which DIPPEL (1950) and HANSON (1956, 1957) have used in cases of mixed populations of the mutant forms of kappa.

The significance of these various cases, including that of "sex-ratio" in *D. willistoni*, lies in the contribution they make toward a clearer understanding of the interactions of the various elements of the genetic systems which are responsible for transmission and expression of the pattern of development from generation to generation.

SUMMARY

1. The "sex-ratio" condition, which has its origin in a single female of *D. willistoni* found in nature and subsequently maintained in a series of genetically derived strains, has been transferred by injection into adult females of a wild strain, Recife-3, of the same species previously free of this condition and producing normal proportions of the two sexes.

2. The unisexual progenies characterizing the "sex-ratio" strain arise through the action of an agent which is transmitted by the females to all their eggs and is lethal in all XY zygotes.

3. Ooplasm from the lethal XY zygotes provides the effective agent which when transferred into the abdomens of females of previously normal strains may give rise to intermediate or unisexual broods among their progeny.

4. The "sex-ratio" condition does not appear immediately in the progeny of injected females, but follows a latent period of 10–14 days (in those cases in which it is expressed) as demonstrated by analysis of successive two day broods.

5. The "sex-ratio" condition is not immediately stabilized in the host strain for many females from unisexual broods fail to transmit the condition and some produce intermediate progenies. In certain instances unisexual progenies made their appearance in subsequent generations from the daughters of bisexual progenies.

6. Stabilized unisexual strains derived from several of the injected females have been established and one has been maintained for 14 generations.

7. The wild strain, Recife-3, in which the artificially produced unisexual conditions have been established is genotypically unfavorable for the persistence of the "sex-ratio" agent indicating that adaptation, presumably involving mutation and selection of the agent, occurred during the course of stabilization.

8. The findings are discussed in relation to other cases of cytoplasmic inheritance.

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RELEASE OF GENETIC VARIABILITY THROUGH RECOMBINATION. III. DROSOPHILA PROSALTANS

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THIS is the third in a series of articles on release of genetic variability through recombination in three species of Drosophila. The previous articles have dealt with *Drosophila pseudoobscura* (SPASSKY *et al.* 1958) and *D. persimilis* (SPIESS 1959). The present article is concerned with *D. prosaltans*. The ecological peculiarities of this species are pertinent. It is native exclusively in the tropics, while the other two species live mainly in the temperate zone. *D. pseudoobscura* is widely distributed, very common, ubiquitous, and ecologically versatile; *D. persimilis* occurs in a much less extensive geographic area, and although it builds very dense populations in favorable habitats it is specialized to live chiefly in cool and humid locations; *D. prosaltans* is a rare form, which reaches considerable population densities only ephemerally in few scattered neighborhoods (DOBZHANSKY and PAVAN 1950). The spontaneous mutation rates for autosomal lethals are, at similar temperatures and in homologous chromosomes, about twice as high in *D. persimilis* and *D. prosaltans* as they are in *D. pseudoobscura* (DOBZHANSKY, SPASSKY and SPASSKY 1952, 1954). The genetic loads carried in the populations (accumulated recessive lethal, semilethal, and subvital gene complexes in the chromosomes) seem nevertheless to be higher in *D. pseudoobscura* than in *D. persimilis* or in *D. prosaltans* (DOBZHANSKY and SPASSKY 1953, 1954). In accordance with this, the loss of fitness produced by inbreeding and homozygosis for naturally occurring gene complexes is greater in *D. pseudoobscura* than in *D. persimilis* or in *D. prosaltans*. It is tempting to speculate that the adaptive norm of *D. pseudoobscura* depends upon balanced heterozygosis to a greater extent than is the case in *D. persimilis* and in *D. prosaltans*. In other words, the genetic architecture of *D. pseudoobscura* approaches that postulated by the "balance" hypothesis, while the genetic architectures of the other two species incline relatively more towards the situation envisaged by the "classical" hypothesis (DOBZHANSKY 1955).

Material and technique in Drosophila prosaltans

The experiments have followed the same plan as those with *D. pseudoobscura* and *D. persimilis* (SPASSKY *et al.* 1958, SPIESS 1959). Ten second chromosomes, permitting normal or subnormal viability in homozygotes, were extracted from

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a sample of *D. prosaltans* collected in May, 1954, at Pirassununga, in the state of São Paulo, Brazil, by PROF. C. PAVAN and kindly sent by him to our laboratory. These chromosomes will be referred to below, for brevity, as "Pira" chromosomes. Another set of ten normally viable to subvital second chromosomes was extracted from the sample of flies kindly collected for us in April, 1954, in the vicinity of Rio de Janeiro by DRS. H. BURLA and C. MALOGOLOWKIN. These chromosomes will be referred to as coming from "Rio." The techniques of testing the chromosomes of *D. prosaltans* for viability effects have been described by DOBZHANSKY and SPASSKY (1954). The 20 strains containing the original chromosomes used in the present experiments (ten Pira and ten Rio strains) were kept in homozygous condition in a constant temperature room at 19° C.

All the possible intercrosses of the 20 strains were made. Females carrying a given pair of the original chromosomes were then outcrossed to males which carried in one of their second chromosomes the dominant mutant Lobe (eye shape, see SPASSKY, ZIMMERING and DOBZHANSKY 1950). Ten Lobe males were taken from each progeny, and crossed, in individual cultures, to females which carried in one of their second chromosomes the dominant mutants Plum, Star, and Curly (*Pm S Cy*). In each progeny, about five pairs of females and males showing the effects of the genes *Pm*, *S*, and *Cy* were selected and inbred. In the next generation, exactly 100 flies were counted in each progeny, and the numbers of *Pm S Cy* and wild type flies were recorded. The *Pm S Cy* chromosome carries a complex inversion which suppresses most of the crossing over. All the cultures in which counts were made were raised at 25° C.

Viability of the original and the recombination chromosomes

The experimental data are summarized in Tables 1–4, which are constructed exactly like the comparable Tables for *D. pseudoobscura* and *D. persimilis* (SPASSKY *et al.* 1958, SPIESS 1959). The strain numbers are shown on the top and the left margins of the tables. Roman type above and to the right of the diagonals in Tables 1 and 2, and all the figures in Table 3, are the mean numbers of wild type (non-Plum-Star-Curly) flies in the test cultures of the recombination chromosomes. Italic type numerals below and to the left of the diagonals in Tables 1 and 2, and all the figures in Table 4, give variances of the numbers of wild type flies in the ten cultures testing viabilities in homozygous condition of the recombination products of a given pair of original chromosomes. The figures on the bottom and the right margins in the four tables are the mean viabilities, or the mean variances, of the recombination products in which a given original chromosome has participated. Finally, the squares along the diagonals in Tables 1 and 2 give the mean viabilities (Roman) and variances (italics) of the homozygotes for the ten original chromosomes from Pirassununga and from Rio de Janeiro. The original chromosomes were tested (or, rather, retested) at the conclusion of the experiments on the recombination products, each original chromosome in sextuplicate, and the figures in the squares along the diagonals are based on the data obtained in these retests. All the variances are the residual variances

(V), obtained by subtracting the binomial sampling error from the crude variance *(U)*.

The mean viability of the homozygotes for the ten original Pira chromosomes (Table 1) is 30.53, and for the ten original Rio chromosomes (Table 2) 33.11. According to DOBZHANSKY and SPASSKY (1954), the mean viability of the homozygotes for a random sample of second chromosomes extracted from natural populations of *Drosophila prosaltans* is 21.3, while the normal (control) viability

TABLE 1

Viability (Roman type numerals above and to the right of the diagonal line) and variance (italic numerals below and to the left of the diagonal) of the recombination products of ten original chromosomes (nos. 13, 15 . . . 55) from Pira. $\overline{M}$ *and* $\overline{V}$ *= mean viability and mean variance of the recombination products of the crosses in which a given original chromosome is a participant. The squares along the diagonal show the viability (Roman) and the variance (italic) of the original chromosomes. Further explanation in text*

	13	15	48	49	68	69	72	78	82	55	$\overline{M}$
13	29.5 *−0.1*	28.9	30.0	29.0	23.0	30.0	27.8	32.0	26.1	30.7	*28.6*
15	*0.4*	32.3 *24.8*	30.5	26.9	28.1	29.7	31.7	33.0	18.9	28.2	*28.4*
48	*40.1*	*26*	23.7 *9.5*	25.8	27.5	31.2	29.2	31.2	27.9	28.7	*29.1*
49	*51.2*	*−4.0*	*14.6*	32.3 *−16.4*	29.2	27.7	13.3	26.1	31.3	20.1	*25.5*
68	*−2 2*	*30 6*	*33 2*	*−3 8*	32.3 *−8.0*	28.3	29.2	25.6	28.3	27.5	*27.0*
69	*13.9*	*58.2*	*10.9*	*23.8*	*35.0*	34.0 *−11.6*	27.7	29.4	31.9	16.2	*27.4*
72	*21.9*	*21.9*	*9.1*	*198.9*	*−6.7*	*36.4*	35.0 *16.8*	25.3	30.5	32.9	*28.0*
78	*16.9*	*28.1*	*10.9*	*170.4*	*16.1*	*−1.2*	*22.9*	29.8 *28.4*	29.7	29.4	*27.5*
82	*11.5*	*160.1*	*27.8*	*97.6*	*14.4*	*7.5*	*12.6*	*50.5*	23.8 *−8.4*	29.1	*29.1*
55	*14.1*	*−0.9*	*35.6*	*204.9*	*44.8*	*49.9*	*28.0*	*8.8*	*24.4*	32.5 *21.6*	*28.2*
$\overline{V}$	18.6	33.0	20.5	83.7	17.9	26.0	38.3	35.9	45.2	45.5	

is 33.85. The original chromosomes utilized in the present experiments are, thus, normally viable to mildly subvital in homozygotes.

The mean viability of homozygotes for the recombination products of these original chromosomes is markedly lower than that of the original homozygotes themselves. The figure for the recombinations of the Pira chromosomes (grand mean) is 27.88, for the Rio chromosomes 28.12, and for the interpopulational crosses (Pira × Rio) 28.27. It is evident, then, that in *D. prosaltans*, as in *D. pseudoobscura* and *D. persimilis*, the recombination between chromosomes which give homozygotes of better-than-average viability leads to depression of the recombinants towards the mean viability of the homozygotes for randomly chosen chromosomes in natural populations of the species. It is interesting to note

TABLE 2

Viability and variance of the recombination products of ten original chromosomes for Rio. For further details see the legend to Table 1 and text

	22	90	105	118	122	127	142	158	190	232	$\bar{M}$
22	30.6 -15.6	23.3	24.2	37.3	31.4	23.1	27.0	30.4	27.2	25.8	28.0
90	148 8	35.0 -10.0	33.9	34.4	32.4	30.7	34.9	34.5	11.0	21.4	28.5
105	17.8	38.6	33.0 -8.1	34.9	17.7	24.8	32.7	36.0	16.1	26.8	27.5
118	38.2	41.9	-5.7	37.8 -4.6	19.1	31.8	31.3	31.8	29.3	32.0	31.3
122	74.1	-13.4	269.0	312.0	31.2 -16.8	20.8	31.4	25.9	29.7	29.1	26.7
127	109.0	-1.0	103.3	18.7	200.2	36.3 2.1	34.6	33.5	25.6	33.3	28.7
142	18.5	1.6	0.2	11.0	71.6	32.8	33.0 -10.9	29.7	17.4	28.0	29.7
158	130.2	47.0	0.1	-14.4	47.1	-10.9	3.8	33.3 0.8	22.2	27.4	30.7
190	56.8	200.1	378.8	32.2	22.7	15.7	322.0	40.7	30.3 48.3	27.0	22.8
232	-2.5	154.8	69.2	13.8	34.8	-8.4	4.1	31.5	-8.6	30.5 -16.9	27.9
$\bar{V}$	65.7	68.7	96.8	49.7	113.1	51.0	51.7	30.6	117.8	32.1	

that this regression is apparently less pronounced in *D. prosaltans* and in *D. persimilis* than in *D. pseudoobscura* (see below).

Variance engendered by recombination

The mean residual variance *(V)* for the original chromosomes turns out to be 5.6 for the Pira chromosomes, and –3.6 for the Rio chromosomes. In *D. prosaltans*, like in the other species, this residual variance is contributed to by the environmental differences between the six replicate test cultures, and by the possible accumulation of mutations in the strains in which the original chromo-

TABLE 3

Viability of the recombination products of chromosomes from Pira and from Rio. $\overline{M}$ *= the mean viability of the recombination products of the crosses in which a given original chromosome is a participant*

		P	I	R	A						
		13	15	48	49	68	69	72	78	82	55	$\overline{M}$
	22	33.3	30.9	27.4	18.2	30.6	25.1	16.7	30.5	30.3	26.2	*26.9*
	90	30.4	33.9	25.9	27.3	31.7	29.7	30.1	31.2	30.4	37.4	*30.8*
	105	34.8	24.4	31.2	32.2	34.6	34.8	35.0	20.6	32.4	29.2	*30.9*
	118	31.0	32.9	34.1	26.1	31.4	32.7	33.5	33.0	33.6	31.0	*31.9*
O	122	26.4	7.6	31.0	20.0	31.3	9.1	24.8	24.2	27.4	29.3	*23.1*
—	127	29.4	34.5	23.3	14.2	29.5	32.9	34.6	31.7	15.8	27.5	*27.3*
R	142	33.3	33.8	23.2	20.5	32.8	34.9	33.6	36.5	33.6	34.1	*31.6*
	158	34.2	35.4	32.2	31.9	28.4	12.9	16.6	36.9	20.3	17.8	*26.7*
	190	35.6	29.9	26.8	25.1	27.2	23.4	28.2	14.4	27.2	27.0	*26.5*
	232	19.0	31.6	30.2	30.6	29.8	30.8	29.5	31.4	26.4	9.6	*26.9*
	$\overline{M}$	*30.7*	*29.5*	*28.5*	*24.6*	*26.9*	*30.7*	*26.6*	*28.3*	*29.0*	*27.7*	

somes were perpetuated during the course of the experiments. It is evident that this residual variance for the original chromosomes is not significantly different from zero.

The variances for the recombination chromosomes is considerable. The mean for the recombination products of Pira chromosomes turns out to be 36.5, and for Rio chromosomes almost twice as large, 67.7. The interpopulational crosses give a variance intermediate between the two groups of the intrapopulational crosses, namely 53.4. Inspections of Tables 1, 2, and 4 shows that some of the original chromosomes seem to yield recombination products more varied in viability than do other chromosomes. Thus, the mean variances of the recombina-

TABLE 4

Variance of the viabilities of the recombination products of chromosomes from Pira and from Rio. $\bar{V}$ = *mean variance in the crosses in which a given original chromosome participates*

	PIRA										
	13	15	48	49	68	69	72	78	82	55	$\bar{V}$
22	24.9	23.2	17.9	17.5	4.1	49.5	225.2	62.9	6.0	24.4	45.6
90	31.1	19.7	76.5	10.8	30.4	9.8	64.2	11.4	19.3	-5.6	26.8
105	17.9	51.6	47.8	31.9	27.9	33.9	3.9	195.2	19.0	4.8	43.4
118	21.7	-4.9	13.4	-1.6	4.3	-14.2	0.2	0.3	17.5	28.2	6.5
0122	0	154.1	13.9	178.4	3.4	154.0	83.3	35.0	23.9	8.2	65.4
— 127	-6.0	-13.7	62.4	231.3	-8.3	-5.5	3.6	-11.9	285.8	113.7	64.9
R 142	17.6	5.4	84.1	66.6	2.4	-16.6	-6.0	11.3	10.2	8.3	18.3
158	25.2	1.8	26.1	16.6	0.4	194.9	310.2	3.7	336.5	341.7	125.7
190	18.7	38.7	33.2	51.1	155.3	153.0	97.9	172.2	45.5	24.5	79.0
232	169.0	36.2	10.2	4.4	32.6	18.2	-1.4	122.3	27.5	170.5	59.0
$\bar{V}$	32.0	31.2	38.6	60.7	25.2	57.7	78.1	60.0	79.1	71.9	

tion products in which the chromosomes Pira-13 and Pira-68 participate are 18.6 and 17.6 (Table 1), while Rio-122 and Rio-190 yield respectively 113.1 and 117.8 (Table 2). Since the residual variance for the original chromosomes is very low, almost the entire variance observed among the recombination products is engendered by recombination.

The release of variability

The statistical analysis of the data for *Drosophila prosaltans* is given in Tables 5 to 8. The organization of these tables is the same as that in the papers dealing with *D. pseudoobscura* and *D. persimilis*, and does not require any special commentary. Accordingly, in this section and the following one the results for all three species will be compared.

The real residual variability as given by $\overline{V}$ for the original chromosomes is in all cases negligible, with a grand average for all three species of 2.4. The variability released by crossing over can be estimated either from $\overline{V}$ for the crosses or from the component of variance within crosses, which differs from $\overline{V}$ only by the subtraction of the negligible $\overline{V}$ for the original chromosomes. For *D. prosaltans* the value of $\overline{V}$ is 68 for the Rio intrapopulational crosses, 36 for the Pira intrapopulational crosses, and the intermediate value of 54 for the interpopulational crosses. The problem of interpretation of such tests as that for the difference between the two intralocality crosses is discussed in the fourth paper of this series (LEVENE 1959). For testing whether the two particular sets of chromosomes used differ significantly, the F ratio of the two sets of $\bar{U}$'s is 1.53 which is highly significant. However, for testing whether there are real differences between the localities themselves a Student's t test is more appropriate and gives a P value of about 0.05.

Taking *D. pseudoobscura* and *D. persimilis* alone, there would seem to be a very significant difference between the amount of recombination obtained. However, we must consider this in the light of the difference found betweeen the two localities for *D. prosaltans*. It should be noted that *D. prosaltans* from Rio gives a $\overline{V}$ closely comparable to that for *D. pseudoobscura*, whereas *D. prosaltans* from Pira is comparable to *D. persimilis*. Accordingly it seems evident that the essential thing is which particular localities were chosen for each species and the rigorous test for differences between species would then be an F test based on two observations (two intrapopulational crosses) for each species. It is not clear how the interpopulational crosses could be used in this test since they do not represent an independent third locality; but since the interpopulational crosses give results usually intermediate between the two intrapopulational crosses, it seems better to ignore them for the present purpose. The test using the intralocality crosses gives $F = 3.53$ with 2 and 3 degrees of freedom which is not significant, P being between 10 and 25 percent.

Results of the analysis of variance

The results of the analysis of variance are given in Tables 5 and 6, of which Table 6 is the more interesting. We will first discuss the analysis of variance for

the means. For *D. pseudoobscura*, there is, in every case, a moderate but significant additive component between crosses, and a somewhat larger, also significant, nonadditive component between crosses, but the total between crosses variance component is only about one third the size of the within crosses component. On the other hand, for *D. prosaltans* none of the additive between crosses components

TABLE 5

Analyses of variance

Description	d.f.	On means F	P (%)	On variances F	P (%)
		Pirassununga intrapopulational cross			
Main effect	9, 35	0.69	50 –75	0.70	50 –75
Interaction	35,405	3.11	⩽0.1	3.14	⩽0.1
Int. of int.	9, 35	1.26	25 –50	2.29	2.5 – 5
Within	405, 50	2.19	⩽0.1	...	.....
		Rio intrapopulational cross			
Main effect	9, 35	1.72	10 –25	1.06	25 –50
Interaction	35,405	3.63	⩽0.1	5.60	⩽0.1
Int. of int.	9, 35	0.70	50 –75	4.28	0.05– 0.1
Within	405, 50	4.26	⩽0.1	...	.....
		Interpopulational cross			
Pira. main	9, 81	0.94	50	0.47	75 –90
Rio main	9, 81	2.18	2.5– 5	1.65	10 –25
Interaction	81,900	5.25	⩽0.1	5.00	⩽0.1
Pira. int. of int.	9, 81	0.36	90 –95	1.54	10 –25
Rio int. of int.	9, 81	0.40	90 –95	0.81	50 –75
Within	900,100	3.44	⩽0.1	...	.....

are significant; but the nonadditive components are somewhat larger so that the total variance between crosses is about the same as in *D. pseudoobscura*. On the other hand the within crosses component for Rio is about the same as *D. pseudoobscura*, while for Pira this component is much smaller. Finally, for *D. persimilis* only one of the additive components of variance is significant, and even that one is extremely small, while the nonadditive components are comparable in size to those obtained for *D. pseudoobscura*. Consequently the total between crosses variance for *D. persimilis* is somewhat smaller than that for the other two species. Futhermore the component within crosses is relatively even smaller and is on the average only about twice the component between crosses. The real residual component of variance is, of course, small for all localities tested, while the binomial component, being a function of the mean, whose total variation for the crosses is only between about 20 percent and 30 percent, is more or less constant throughout. The component of variance between chromosomes for the original chromosomes has no biological meaning since these chromosomes were chosen arbitrarily, but has importance only for purposes of comparison with the additive between crosses components. These two components are usually of comparable

TABLE 6

Components of variance for means, M, *and read variances,* V, *bold face figures are estimates. Figures above and below them are approximate upper and lower 95 per cent confidence limits respectively. Values of within crosses and residual components for* V *are for comparison only (see text). All values of* V *have been divided by 100. All values have two significant figures*

	Description	Additive between crosses	Non-additive between crosses	Total between crosses	Within crosses	Total genetic	"Real" residual	Binomial sampling	Total residual
MEANS (*M*)	Intra P	2.9 **0** 0	24 **12** 5.8	.. **12** ..	41 **31** 16	.. **43** ..	20 **5.6** 0	22 **20** 18	40 **26** 18
	Intra R	19 **2.8** 0	45 **23** 12	.. **25** ..	80 **66** 52	.. **91** ..	6.6 **0** 0	23 **20** 18	27 **20** 18
	P	8.3 **0** 0	 46	.. .. ..	 60	.. .. ..	.. .. 8.8	 22	 29
	Inter	——— **31** 24	**36** 21	**52** ..	**88** 43	**1.0** ..	**20** 0	**21** 18	18
	R	**4.5** 0	.. ..	.. ..	.. ..	.. ..	.. ..	.. ..	.. ..
	Orig P	44 **10** 2.1	.. .. ..	.. **10** ..	.. .. ..	.. **10** ..	20 **5.5** 0	23 **21** 19	41 **27** 19
	Orig R	19 **3.1** 0	.. .. ..	.. **3.1** ..	.. .. ..	.. **3.1** ..	6.6 **0** 0	23 **22** 21	29 **22** 21
VARIANCES (*V*)	Intra P	13 **2.0** 0	31 **15** 7.4	.. **17** ..	8.5 **5.6** 3.5	.. **23** ..	.. .. ..	.. .. ..	3.6 **1.5** 0.75
	Intra R	30 **1.0** 0	140 **76** 44	.. **77** ..	22 **16** 11	.. **93** ..	.. .. ..	.. .. ..	1.6 **0.91** 0.70
	P	7.8 **0** 0	 71	.. .. ..	 14	.. .. ..	.. .. ..	.. .. ..	.. .. 1.9
	Inter	——— **48** 33	**54** 33	**11** ..	**65** 8.0	.. ..	.. ..	.. ..	**1.0** 0.76
	R	**5.7** 0	.. ..	.. ..	.. ..	.. ..	.. ..	.. ..	.. ..
	Orig P	6.6 **0** 0	.. .. ..	.. **0** ..	.. .. ..	.. **0** ..	.. .. ..	.. .. ..	6.9 **2.9** 1.5
	Orig R	10 **1.7** 0	.. .. ..	.. **1.7** ..	.. .. ..	.. **1.7** ..	.. .. ..	.. .. ..	3.3 **2.0** 1.7

size except in a few cases where the variation between original chromosomes is greater than the additive variance for the derived chromosomes.

The comparability of the variance component between chromosomes for the original chromosomes and the additive component between chromosomes for the crosses suggests that the correlations between the means for chromosomes under different conditions be examined. Table 7 gives these correlations for *D. pro-*

TABLE 7

Correlation between the means in different experiments for Pirassununga (above the diagonal) and Rio (below the diagonal) chromosomes

	Original chromosomes	Intra-populational	Inter-populational
Original chromosomes		—0.46	—0.15
Intrapopulational	0.67		0.65
Interpopulational	0.59	0.49	

saltans. The general picture of these correlations is the same for all three species, but does depend somewhat on the kind of comparison being made. Accordingly, all the correlations for the six localities (two for each species) have been combined using Fisher's z transformation. The average correlation of means of the original chromosome with their effects in intrapopulational crosses is 0.03 with confidence limits −0.24 and +0.29. The average correlation of the means of the original chromosomes with their effects in interpopulational crosses is 0.26 with confidence limits of 0 and 0.49, while the average correlation between the additive effects of chromosomes in intra- and interpopulational crosses is 0.57 with confidence limits of 0.37 and 0.73. The average of all the correlations is 0.30 with confidence limits of 0.16 and 0.47. The difference between the correlation of the original with intra- and the correlation of the original with the interpopulational crosses is not significant, but each of these is significantly different from the correlation between the intra- and interpopulational crosses. Nevertheless, even this latter correlation is not very large. Thus the general picture is that there is very little connection between the way a chromosome behaves originally and its average effect in crosses with other chromosomes, but that there is a greater, though not very striking, relationship between the way a chromosome behaves in intra- and interpopulational crosses.

We come now to the analysis of variance of the variances, V. The additive between crosses variance is small and not significant, except for the effect of White Wolf in intrapopulational crosses and of South Fork in interpopulational crosses in *D. persimilis*, and a significant effect of moderate size for Texas in intrapopulational crosses for *D. pseudoobscura*. On the other hand, there is a significant nonadditive component of variance between crosses, of moderate to large size, for every cross except the Texas intrapopulational cross for *D. pseudoobscura*. In other words, there are substantial differences in the amount of variability released for different crosses, but there is no particular pattern to these differences.

The observed differences are considerably larger than could be explained merely by the sampling variability of the estimates themselves.

Lethal recombination chromosomes

Some of the recombination chromosomes, both in the intra- and in the interpopulational crosses, were lethal in double dose. A list of the crosses which produced such lethals, with numbers of the lethal chromosomes in each cross which yielded them, is given in Table 8. A test culture which contained a lethal chromosome produced, of course, 100 *Pm S Cy* flies and no wild type flies. Table 8 shows also the mean viability (the mean percentages of wild type flies) of the recombination chromosomes which did *not* act as lethals, and the variances of these viabilities. These figures may be compared with those in Tables 1–4, which show viabilities and variances of recombination chromosomes *including* the lethals.

TABLE 8

Numbers of synthetic lethal chromosomes obtained in different crosses, and means and variances of viability (percentages of wild type flies) in cultures containing nonlethal chromosomes

P = Pirassununga, R = Rio

Cross	Lethals	Viability Mean	Viability Variance	Cross	Lethals	Viability Mean	Viability Variance
P 15 × P 82	3	27.0	—12	P 49 × R 122	3	28.6	—14
P 49 × P 55	3	28.7	22	P 49 × R 127	5	28.4	23
P 49 × P 72	5	26.6	12	P 55 × R 127	1	30.6	24
P 49 × P 78	1	29.0	98	P 55 × R 158	5	35.6	—13
P 55 × P 69	1	18.0	20	P 55 × R 232	5	24.0	58
R 22 × R 127	1	25.7	49	P 68 × R 190	1	30.2	73
R 22 × R 158	1	33.8	20	P 69 × R 122	6	22.8	55
R 90 × R 190	6	27.5	7	P 69 × R 158	5	25.8	29
R 90 × R 232	2	26.8	37	P 69 × R 190	2	29.3	4
R 105 × R 122	4	29.5	72	P 72 × R 22	4	27.8	38
R 105 × R 127	1	27.6	32	P 72 × R 122	1	27.6	9
R 105 × R 190	5	32.2	213	P 72 × R 158	4	27.7	196
R 118 × R 122	4	31.8	81	P 72 × R 190	1	31.3	1
R 122 × R 127	3	29.7	—5	P 78 × R 105	1	22.9	161
R 142 × R 190	5	34.8	—7	P 78 × R 190	4	24.0	37
P 13 × R 232	3	27.1	—1	P 78 × R 232	1	34.9	2
P 15 × R 122	7	25.3	33	P 82 × R 127	5	31.6	27
P 48 × R 127	1	25.9	—4	P 82 × R 158	4	33.8	63

In all, 45 out of 900 recombination chromosomes in the intrapopulational, and 64 out of 1000 in the interpopulational crosses, were lethal in double dose; 15 intrapopulational and 20 interpopulational crosses produced at least one lethal. With all the crosses combined, we have 5.7 percent lethals among the 1900 chromosomes tested, and 18 percent of the crosses giving at least one lethal recombinant.

The question now arises as to whether all the observed lethals can be explained

as point mutations or whether some, at least, must be synthetic lethals arising purely by recombination between nonlethal chromosomes. For *D. pseudoobscura* synthetic lethals were proven by an excess of crosses with two or three lethal recombinant chromosomes. This was very improbable if all the lethals were point mutations, but *could* occur with synthetic lethals, since the percentage of synthetic lethal derived chromosomes in a given cross is not subject to hard and fast rules.

The distribution of crosses with lethal chromosomes for *D. prosaltans* is 12 crosses with one lethal, two with two, five with three, six with four, seven with five, two with six, and one with seven lethals, out of a total of 190 crosses. This distribution does not differ significantly from what could be expected from point mutations with a suitable mutation rate; on the other hand there is nothing to prevent such a distribution if, in fact, some of the lethals were synthetic. From this data an estimate of about 0.01 for the point mutation rate per generation can be obtained. Since most of this mutation would be occurring at 19°C, this estimate is quite high compared to the estimate of 0.0047 at 25°C obtained by DOBZHANSKY, SPASSKY and SPASSKY (1952). Such a comparison between experiments carried out at different times and under different conditions must be viewed with caution, but it suggests that synthetic lethals may be contributing to the apparent excess of lethals in the present experiment.

For *D. persimilis* very few lethals were observed, and thus little could be said about their distribution; however the fact that so few lethals were observed suggests that there were few if any synthetic lethals in this species, particularly as DOBZHANSKY, SPASSKY and SPASSKY (1954) found a spontaneous mutation rate in this species at 25°C of 0.013, the highest of the three species.

Finally it may be noted that there is still other evidence for the presence of synthetic lethals in *D. prosaltans*. If a chromosome produces many lethal recombination products when it undergoes crossing over with other chromosomes, it seems simplest to suppose that the strain in which this chromosome is perpetuated has acquired a lethal by point mutation. Yet even such lethals may be shown to be synthetic. Table 8 shows that the chromosome Rio-122 of *D. prosaltans* has yielded 28 lethal recombination products in seven out of the 19 crosses in which it participated; one of the crosses, Rio 122 × Pira 15, gave seven lethal chromosomes out of the ten tested. It was suspected that the Rio-122 strain carries a recessive lethal mutant. Flies from this strain were, accordingly, crossed to Lobe and Plum Star Curly flies, a new viable homozygous Rio-112 strain was isolated, and all the interpopulational Pira × Rio-112 crosses were repeated. The numbers of lethal recombination products out of the ten examined in the original test and in the retests were as follows:

Cross	*Test*	*Retest*	*Cross*	*Test*	*Retest*
Pira 13 × Rio 122	6	0	Pira 69 × Rio 122	6	0
Pira 15 × Rio 122	7	7	Pira 72 × Rio 122	1	0
Pira 48 × Rio 122	1	0	Pira 78 × Rio 122	0	0

Pira 49 × Rio 122	3	6	Pira 82 × Rio 122	0	0
Pira 68 × Rio 122	0	4	Pira 55 × Rio 122	0	2

Since the retests were made immediately following the reisolation of the homozygous viable chromosome Rio-112, the 19 lethal recombination products must certainly be synthetic lethals. PROFESSOR C. PAVAN informs us that he had a similar situation in a strain of *D. willistoni*, which gave numerous recombination lethals in the original tests and also in retests.

DISCUSSION

For convenience of reference for this discussion the main results already given in SPASSKY *et al.* (1958), SPIESS (1959), and in the present paper, as well as some additional quantities which will be discussed below are summarized in Table 9. According to the classical theory of population structure (see discussion in DOBZHANSKY 1955) most loci will possess a typical or "wild type" allele and, in addition, certain chromosomes may contain one or a few major mutant alleles which are usually deleterious. Under such conditions when two chromosomes of high viability, presumably containing "good" genes, are crossed, the resulting recombination chromosomes should also have good viability. Another model might be one in which chromosomes contain a great many polygenes which have mostly additive effects. Under such conditions two chromosomes having polygenes that give them reasonably good viability will yield upon recombination

TABLE 9

Mean viabilities and variances of homozygotes for chromosomes of three species. "Natural" refers to means and variances for samples of wild chromosomes obtained directly from natural populations. "Recombination" refers to mean viabilities and variances (V) for chromosomes which were recombination products of pairs of quasi-normal original chromosomes obtained from nature

Species and chromosomes	Mean viability		Variance		Recombination: Natural variance (%)	
	All chromosomes	Quasi-normal	All chromosomes	Quasi-normal	All chromosomes	Quasi-normal
D. pseudoobscura						
Natural	20.29	24.26	140	65	43	74
Recombination	22.92	23.93	60	48		
D. persimilis						
Natural	23.54	28.00	110	60	24	27
Recombination	28.16	28.76	26	16		
D. prosaltans						
Natural	21.17	25.98	200	85	25	28
Recombination	28.14	29.72	50	24		

chromosomes with a large array of different viabilities. However, if the effects are additive, the mean viability of all recombination chromosomes should be the same as the average of the viabilities of the two original chromosomes. Two types of interaction can change this picture, dominance, and epistasis. Since in the present case we are dealing with chromosomes in the homozygous state, only epistasis can play any part. It was pointed out in the discussion of the first paper (SPASSKY *et al.* 1959) that the nonadditive component of variance for the means represents such epistatic interaction. It has been suggested by LEWONTIN (personal communication) that the appearance of the nonadditive effect may be due to the use of the wrong scale of measurement. He suggests that viability may be multiplicative rather than additive, and that the use of the logarithm of viability rather than viability itself would be more appropriate. While this argument has a certain appeal, the use of a logarithmic transformation in our data would accentuate the nonadditivity rather than remove it. Additivity, by its very nature, can only refer to some particular scale of measurement, and in many genetic experiments, it is possible to obtain additivity by a suitable monotonic transformation. With complete additivity, the mean for recombinant chromosomes would be exactly halfway between the means for the two parental chromosomes; if this mean lay between the two parental values but not at the half way point, it might be possible to find a suitable transformation that would bring it to the half way point. However, in the present data, not only is the mean for the recombinant chromosomes very much below the mean for either set of parental chromosomes but the same is true for the median, and no monotonic transformation could bring this median to a point between the two parental medians. This is the strongest kind of evidence that epistatic interaction plays a major role in natural populations. Evidently the original chromosomes, chosen to have fairly high viabilities when homozygous, carried genes which interacted epistatically in a harmonious way, whereas the random recombination products in general contained genes that are on the average less harmonious in their epistatic interaction. It should, of course, be remembered that in natural populations the gene complexes in the chromosomes are not present in the homozygous state, but in a heterozygous state and the present evidence does not permit evaluation of the relative importance which dominance interaction and epistatic interaction in heterozygotes may have in determining a quantitative character, such as the kind of viability studied here.

The diversity of viability effects among chromosomes resulting from recombination of genes carried in pairs of apparently similar original chromosomes is very great. It is of interest to compare the variance which arises from such recombination with the total variance observed between different chromosomes obtained in nature. The distribution of viabilities for the Texas (*D. pseudoobscura*) chromosomes, of which the ones in the present study are a subsample, is given in DOBZHANSKY, PAVLOVSKY, SPASSKY and SPASSKY (1955), the distributions for *D. pseudoobscura* and *D. persimilis* from California are given in DOBZHANSKY and SPASSKY (1953), and those for *D. prosaltans*, from localities other than those

presently under study, are given in DOBZHANSKY and SPASSKY (1954). The mean viabilities and variances for these distributions have been calculated in two ways, namely for all chromosomes studied and for quasi-normal chromosomes (i.e., excluding chromosomes which were lethal or semilethal to homozygotes). The results are shown in Table 9. These two papers have reported the observed variances for quasi-normal chromosomes, and also the total residual variances. By subtracting the total residual variances from the crude variances, we obtain the "real" or "genetic" variances, corresponding to the components of variance arising through recombination within the crosses described in the present series of papers on *D. pseudoobscura*, *D. persimilis*, and *D. prosaltans*.

Table 9 also gives the ratio of the variance within chromosomes obtained by recombination in the present experiments to the variance of "natural" chromosomes, expressed as a percentage. This ratio varies from 24 percent and 25 percent (for all chromosomes in *D. persimilis* and *D. prosaltans*) to 74 percent (for quasi-normal chromosomes in *D. pseudoobscura*). This is a very remarkable result. We have selected as our "original" chromosomes groups of chromosomes which yield homozygotes of quasi-normal viability, in fact on the average above the mean viability of a random sample of quasi-normal chromosomes found in nature. And yet, we find that recombination of the gene contents of these chromosomes produces an amount of genetic variability that is a substantial fraction, between one quarter and three quarters, of the total genetic variability found among all chromosomes in natural populations. The chromosomes obtained through recombination include, in our experiments, the whole range of viabilities, from lethal, semilethal, through subvital, normal, and supervital chromosomes.

The above observations throw some light on one of the basic problems of evolutionary genetics, namely that of the mechanisms which maintain the immense genetic diversity which we find in the natural populations of sexually reproducing species. According to the classical theory of population structure, this diversity is, in any one environment, due primarily to the presence of more or less recently arisen mutants which have not yet been eliminated by natural selection. The data described in the present series of three articles militate against the classical theory. The genetic diversity which we find in the populations is evidently exceeded by the concealed or potential variability stored in the linked gene complexes in naturally occurring chromosomes, which can be released by recombination.

Indeed, the variance which is released in one generation by recombination of parts of chromosomes selected for a relative uniformity amounts to at least one quarter of the total expressed variance. The fact that some of the recombination products are semilethal or lethal when homozygous is particularly illuminating. The frequency of such synthetic lethals will be maintained in a population not by newly arising mutations, but rather by an equilibrium between the frequencies of their being "synthesized" and "desynthesized". Such a situation is compatible with the balance theory of population structure (DOBZHANSKY 1955). The genetic diversity is maintained primarily not by new mutants, but by the advantages of

heterozygosis for gene alleles and gene complexes which are kept up by natural selection, and also by environmental fluctuations in space and in time which alter the signs and the magnitudes of selective advantages and disadvantages. With a population structure of this sort, a total suppression of the mutation process would probably fail to change the evolutionary plasticity of the species for very many generations.

How widespread is the genetic population structure of the sort we have found is quite another matter. Even the three Drosophila species investigated are perceptibly, and meaningfully, different in this respect. It can be seen in Table 9 that the ratio of the variance released by recombination to the total natural variance observed in nature is higher in *D. pseudoobscura* than it is in *D. persimilis* and *D. prosaltans*. As stated in the introduction, *D. pseudoobscura* is a far more common, successful, and versatile species than *D. persimilis*, and especially than *D. prosaltans*. It has the greatest amount of potential genetic variability stored in the chromosomes, and presumably approaches most closely the population structure visualized by the balance theory.

The fact that the ratio of recombination : total variance is apparently equal in *D. persimilis* and *D. prosaltans* may seem unexpected. The latter species has also a higher expressed natural variance than *D. persimilis* and even than *D. pseudoobscura*. The answer is that the second chromosome of *D. prosaltans* used in the experiments is not a homologue of the second chromosomes of the two other species, and, contains, in fact, almost twice as much chromatin as the latter (SPASSKY, ZIMMERING and DOBZHANSKY 1950). The data for *D. prosaltans* were meant to be compared with those for the homologous second chromosome of *D. willistoni*, a very common and successful tropical species. Unfortunately, the experiments with the latter were not completed. It may also be noted that the recombination : total variance ratio in *D. pseudoobscura* appears to be higher in the California than in the Texas populations. This may be a reflection of the fact that the Texas population studied came from an extreme margin of the distribution area of the species, while the California population is subcentral.

What is the role of newly arising mutation in the maintenance of the genetic diversity in the populations of the three species studied remains unclear. The lack of reliable data on spontaneous mutation frequencies in polygenic systems is, in general, one of the greatest gaps in our understanding of the mechanisms of origin of raw materials of evolution. DOBZHANSKY and SPASSKY (1947) submitted to severe selection 14 strains of *D. pseudoobscura* propagated in laboratory cultures; the conditions of the experiment were such that the measured success of the selection could depend only on newly arisen mutants. In 50 generations, the selection was effective in 11 out of 14 strains. On the other hand, the evidence on mutation is largely negative in the present experiments. As stated above, 60 chromosomes were tested from the cultures of the original chromosomes at the end of the experiments, after they had had chance to accumulate mutants for some 10–20 generations. In only one instance (in *D. persimilis*) was the presence of a lethal ascertained. More to the point is that the study of the variance leads

to an essentially negative conclusion. As estimated from our data, the variance observed may be the sum of genetic and environmental components, and this sum, although usually ostensibly greater than zero, is quite small in replicate tests of the original chromosomes compared to the variance released by recombination.

Comparisons of the population structures in the species of Drosophila with that in the human species would obviously be devoid of basis at present. It may however be noted that MORTON, CROW, and MULLER (1956) have inferred that the genetic structure of human populations agrees best with that envisaged by the classical theory. Their method of analysis is most ingenious, involving comparison of mortality rates in the offspring of marriages between cousins and other relatives and in the general population. Unfortunately, their methods are based on several assumptions, one of which is that the loss of fitness stemming from homozygosis for genes which are heterotic in heterozygotes is due to genes each represented by only two alleles in the population. This assumption is not a necessary one. In Drosophila populations heterosis results frequently from interaction not between alleles of a single locus but between "supergenes", i.e., linked complexes of polygenes. The present series of three papers has shown that a very great variety of such "supergenes" arise in the chromosomes of every species studied by recombination. Natural selection will, in every population, select those "supergenes" which interact favorably in heterozygous combinations with other "supergenes" present in the same population.

SUMMARY

Like in the experiments of SPASSKY *et al.* (1958) and SPIESS (1959) on *Drosophila pseudoobscura* and *D. persimilis*, two groups of ten second chromosomes of *D. prosaltans* were chosen from population samples taken in two localities, both in southern Brazil. The chromosomes chosen yielded homozygotes of subnormal to normal viability. All possible intercrosses (190) between the strains carrying the 20 original chromosomes were made; from each intercross ten chromosomes, which were probably products of recombination between pairs of the original ones, were taken and tested for viability in double dose. Like in the other species, a great mass of genetic variability is released by recombination of the gene contents of the original chromosomes. The viability of the recombination products in homozygous condition ranges all the way from normal to lethal (synthetic lethals). Much of this variability released by recombination is attributable to epistatic interactions between the different polygenes contained in the original chromosomes.

Comparison of the species suggests that recombination releases more variability in *D. pseudoobscura*, a widespread, common, and ecologically versatile species, than in *D. persimilis* and *D. prosaltans*, which are more specialized forms adapted to probably narrower ranges of habitats. Even so, the amount of the variability released by recombination between pairs of quasi-normal chromosomes is astonishingly great in all three species. In *D. pseudoobscura* it amounts to about 43

percent, and in *D. persimilis* and *D. prosaltans* to about 24 percent and 25 percent of the total variance present in the natural populations from which the original chromosomes were taken (see Table 9). It is concluded that the genetic variance of the viability in natural populations depends only to a slight extent on newly arisen mutants. Most of the variance is due to recombination within the accumulated store of genetic variants.

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RELEASE OF GENETIC VARIABILITY THROUGH RECOMBINATION. IV. STATISTICAL THEORY

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THIS is the fourth of a series of articles on the release of genetic variability through recombination. Identical experiments were performed on three species of Drosophila, and the results were given and analyzed in papers on *D. pseudoobscura* (SPASSKY *et al.* 1958), *D. persimilis* (SPIESS 1959) and *D. prosaltans* (DOBZHANSKY *et al.* 1959). The present paper describes the statistical methods and concepts used in all three analyses, discusses the definition and interpretation of the components of variance involved, and discusses some conceptual difficulties connected with the use of statistical tests of significance on material of this kind.

Model for the analysis of variance

The mathematical model for the analysis of variance of viability for the interpopulational cross is as follows. Let X_{ijk} be the observed viability of the kth derived chromosome from the cross between the ith chromosome of population T and the jth chromosome of population C, where the populations are labelled T and C for definiteness. We suppose that X_{ijk} is the sum of a number of components:

$X_{ijk} = m + t_i + c_j + d_{ij} + w_{ijk} + r_{ijk} + b_{ijk}$, where m is the grand mean and all the other contributions are random effects with zero means and with variances σ_t^2, σ_c^2, σ_d^2, σ_w^2, σ_r^2 and σ_b^2 respectively.

Since the means are zero, if any of these variances is equal to zero the corresponding contribution will be identically equal to zero, and will play no part in determining the value of X_{ijk}. In explaining the meaning of these components it will be convenient to suppose that all components to the right of the one being discussed are equal to zero.

The component t_i represents the additive effect of the ith chromosome from population T, and has the same value for all chromosomes derived from the ith chromosome from population T. Similiarly c_j represents the additive effect of the jth chromosome from population C, and has the same value for all chromosomes derived from the jth chromosome from population C. Thus at this level, the value of X_{ijk} can be completely predicted if we know the grand mean and the additive effects of the chromosomes from the two populations. The component d_{ij} is the nonadditive effect of the cross between the ith chromosome from population T,

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and the jth chromosome from population C, and has a specific value for all chromosomes derived from this cross. Thus, as soon as this component is present, it is no longer possible to estimate the viability of a derived chromosome from the additive effects of the two chromosomes from which it was derived; and in fact d_{ij} measures the departure of the actual viability from this predicted value. The component w_{ijk} is the genetic component within crosses, and represents the difference between the actual viability of the kth derived chromosome from this cross and the mean viability characteristic of chromosomes derived from that cross. Thus σ_w^2 represents the variability released by recombination and is the primary component of interest in the present papers. The sum of the components through w then gives the true genetic viability of a particular derived chromosome, and in principle can be estimated to any desired accuracy by repeated measurements on the same derived chromosome. The component r_{ijk} is the component for the real residual variance and represents the difference between the true genetic viability of a given derived chromosome and its actual viability as affected by the residual genetic variance and, more important, environmental variance. Finally the component b_{ijk} represents the binomial sampling variance due to the fact that we only observed 100 flies.

The variances of the components just discussed are estimated in the components of variance column of Table 3. The components through d_{ij} can be estimated directly from the data on the interpopulational cross, but this data will only give us the sum of the last three components. However, the binomial component is a simple function of the grand mean, and thus can easily be estimated, and the component r_{ijk} includes just those factors affecting the replicate cultures of the original chromosomes, and thus can be estimated from that data.

For certain statistical procedures used it is necessary to assume that the variables entering into X_{ijk} are normally distributed. Now b_{ijk} is a binomial variable and is essentially normally distributed, and r_{ijk}, which mainly represents the effect of enviroment, is probably likewise nearly normally distributed. Furthermore, it seems reasonable to assume that w_{ijk} is normally distributed, except that there are too many extremely small values of w, corresponding to lethality. Again t_i, c_j and d_{ij} can probably be taken to be normally distributed without too much error. Finally X_{ijk}, being the sum of all these components is likely to be still more nearly normally distributed, except that the value zero, denoting lethality, occurs more often than would be expected if X were normally distributed. Eliminating lethals, the X_{ijk} observed are normally distributed to a sufficiently good approximation. Thus the analysis of variance might be more valid if the data were analyzed with lethals eliminated; however, reasons have been given in a previous paper of this series (DOBZHANSKY *et al.* 1959) for preferring to deal with all variation simultaneously. Luckily the F test of the analysis of variance is insensitive to moderate departures from normality and it is felt that it may safely be used on the present data. Estimation of the components of variance themselves does not require any assumption of normality.

The mathematical models for intrapopulational crosses and for the original

chromosomes follow the same lines, and need not be given here in detail. However, it might be mentioned that if the additive effects of the chromosomes in the intrapopulational crosses are denoted as a_1 to a_{10}, each X_{ijk} includes *two* a's, corresponding to the ith and jth chromosomes.

Analysis of variance of means

The analysis of variance for the original chromosomes is as follows. For a given locality let X_{ij} be the viability (number of wild type flies in 100 flies) of the jth replicate culture of the ith original chromosome, let M_i be the mean viability of the 6 cultures of the ith chromosome, and let $\overline{M}$ be the mean of the ten chromosomes. Table 1 gives the analysis of variance, which is an ordinary one-way

TABLE 1

Analysis of variance for original chromosomes

Effect	Sum of squares	d.f.	Mean squares	F ratio	Components of variance
Between chromosomes	$6[\sum M_i^2 - M^2/10]$	9	$\overline{A}$ = (sum of sq)/d.f.	$\overline{A}/\overline{U}$	$(\overline{A}-\overline{U})/6$
Real residual		50	$\overline{V}=\overline{U}-\overline{B}$		$\overline{V}$
Binomial		..	$\overline{B}=\overline{M}(100-\overline{M})/100$		$\overline{B}$
Total residual	$\sum_i[\sum_j X_{ij}^2 - 10M_i^2]$	50	$\overline{U}$ = (sum of sq)/d.f.		$\overline{U}$

analysis of variance with extra lines for the "real residual" and binomial components. It should be noted that this and the other analyses of variance in the present paper are given in the customary form for such analyses. The sums of squares and mean squares in each table are greater by a constant factor (six for the present table) from the form judged simplest for the explanation in the first paper of the series. However the F ratios and components of variance are identical in both forms, and as the sums of squares and mean squares were not given in the tables in the previous papers, no confusion should result. To help construct the present tables, the letters $\overline{A}$, $\overline{C}$, etc. are used to denote mean squares which have no letter assigned to them in the earlier papers.

The analysis of variance for the intrapopulational crosses is as follows. Let X_{ijk} be the viability of the kth derived chromosome for the cross between chromosomes i and j, and M_{ij} be the mean viability for this cross. Let $M_{i.}$ be the sum of the M_{ij} for the nine crosses involving the ith chromosome, $M_{..}=\Sigma M_{i.}$, which is twice the sum of the 45 M_{ij}, let $\overline{M}_i=M_{i.}/9$ be the mean viability for the crosses involving the ith chromosome, and let $\overline{M}=M_{..}/90$ be the grand mean. Also let L^2 equal the sum of the squared values of the 45 M_{ij}; that is $L^2=\sum_{i<j}\sum M^2{}_{ij}$. Also let $U_{ij}=\Sigma(X_{ijk}-M_{ij})^2/9$ and let $\bar{U}$ be the mean of all 45 U_{ij}. Finally, quantities like $\overline{U}$ derived *from the cross in question* will be denoted by the sub-

script $\overline{U}_{intra}$, while corresponding quantities calculated from the data for the *original chromosomes* will be denoted by $\overline{U}_{orig}$. The analysis of variances is given in Table 2. The factor by which the sums of squares and mean squares in this table exceed those discussed in the previous papers is 80. This analysis of variance, except for the lines for the real residual and binomial components, is of the type called a diallel analysis of variance, first discussed by SPRAGUE and TATUM (1942) and corrected by COCHRAN (FEDERER 1951). It is conveniently summarized by GRIFFING (1956).

The analysis of variance for the interpopulational crosses is as follows. For definiteness denote the two populations T and C. Then let X_{ijk} be the viability of the kth derived chromosome for the cross between the ith chromosome from T and the jth chromosome from C, and let M_{ij} be the mean viability of the ten derived chromosomes from this cross. Further let $M_{i.}=\sum_j M_{ij}$ and $M_{.j}=\sum_i M_{ij}$. Then $\overline{M}_{i.}=M_{i.}/10$ is the mean viability for all crosses involving the ith chromosome from T and $\overline{M}_{.j}=M_{.j}/10$ is the mean viability for all crosses involving the jth chromosome from C. Also let $M_{..}=\sum\sum M_{ij}=\sum_i M_{i.}=\sum_j M_{.j}$ be the sum of all 100 M_{ij}, and $L^2=\sum\sum M_{ij}^2$ be the sum of all 100 M_{ij}^2. Then the analysis of variance is given in Table 3. In this table quantities like $\overline{V}_{orig}$ are the *means* of the corresponding values for the original chromosomes in both populations. The factor by which the sums of squares and mean squares in this table exceed those discussed in the previous papers is 100.

The analysis of variance of variances

The model for the analysis of variance of means assumes that the component w_{ijk} has a constant variance σ_w^2 regardless of the cross in question, and that the estimates which could have been obtained individually for each cross would differ only because of sampling error due to the fact that only ten derived chromosomes are studied for each cross. In point of fact it seems more reasonable that different crosses involving different genetic material should also differ in the amount of genetic variability they release. Such variability in the value of σ_w^2, if not too great, does not seriously interfere with the actual carrying out of the analysis of variance for means; nevertheless, it is of some interest to consider this variability for its own sake, and that is done in the present section. The sums of squares for these analyses of variance are obtained exactly as before, except that the values V_{ij} are used instead of the M_{ij} and the factor six in Table 1 and ten in the other two tables, by which the sum of squares is multiplied, is omitted. Similarly, the denominator in the corresponding component of variance is reduced by a factor of six in the first table and ten in the other two tables. The reason for this is that each V_{ij} is a single value whereas M_{ij} is already the mean of either six or ten observed values. Because each cross gives only a single value of V_{ij} it is not possible to obtain an empirical estimate of the variability of the variance within a cross. Nevertheless, it is essential to have some estimate of this variability if we are to tell whether there is a real nonadditive component between crosses or

TABLE 2

Analysis of variance for viability of derived chromosomes for intrapopulational crosses

Effect	Sum of squares	d.f.	Mean squares	F ratios	Components of variance
Additive between	$10[(\Sigma M_{i\cdot}^2)/8 - M_{\cdot\cdot}^2/80]$	9	$\overline{C} = (\text{sum of sq})/\text{d.f.}$	$\overline{C}/\overline{D}$	$(\overline{C} - \overline{D})/80$
Nonadditive between	$10[L^2/2 - (\Sigma M_{i\cdot}^2)/8 + M_{\cdot\cdot}^2/144]$	35	$\overline{D} = (\text{sum of sq})/\text{d.f.}$	$\overline{D}/\overline{U}_{intra}$	$(\overline{D} - \overline{U}_{intra})/10$
Within		405	$\overline{U}_{intra}$	$\overline{U}_{intra}/\overline{U}_{orig}$	$\overline{U}_{intra} - \overline{E}$
Real residual		50	$\overline{V}_{orig}$		$\overline{V}_{orig}$
Binomial		..	$\overline{B}_{intra} = \overline{M}(100 - \overline{M})/100$		$\overline{B}_{intra}$
Total residual		50	$\overline{E} = \overline{V}_{orig} + \overline{B}_{intra}$		$\overline{E}$

TABLE 3

Analysis of variance for viability of derived chromosomes for crosses between two populations designated T and C

Effect	Sum of squares	d.f.	Mean squares	F ratios	Components of variance
Additive between for T	$10\left[(\Sigma M_{i\cdot}^2)/10 - M_{\cdot\cdot}^2/100\right]$	9	$\overline{G} = (\text{sum of sq})/\text{d.f.}$	$\overline{G}/\overline{S}$	$(\overline{G} - \overline{S})/100$
Additive between for C	$10\left[(\Sigma M_{\cdot j}^2)/10 - M_{\cdot\cdot}^2/100\right]$	9	$\overline{H} = (\text{sum of sq})/\text{d.f.}$	$\overline{H}/\overline{S}$	$(\overline{H} - \overline{S})/100$
Nonadditive between	$10\left[L^2 - (\Sigma M_{i\cdot}^2)/10 - (\Sigma M_{\cdot j}^2)/10 + M_{\cdot\cdot}^2/100\right]$	81	$\overline{S} = (\text{sum of sq})/\text{d.f.}$	$\overline{S}/\overline{U}_{inter}$	$(\overline{S} - \overline{U}_{inter})/10$
Within		900	$\overline{U}_{inter}$	$\overline{U}_{inter}/\overline{U}_{orig}$	$\overline{U}_{inter} - \overline{W}$
Real residual		100	$\overline{V}_{orig}$		$\overline{V}_{orig}$
Binomial		..	$\overline{B}_{inter} = \overline{M}(100 - \overline{M})/100$		$\overline{B}_{inter}$
Total residual		100	$\overline{W} = \overline{V}_{orig} + \overline{B}_{inter}$		$\overline{W}$

whether the observed mean square between crosses is just what might be expected due to sampling variability. If we can assume that the underlying data from which the V_{ij} are calculated (the X_{ijk}) are normally distributed we can obtain a theoretical value for this variance as follows.

If $X_1, \ldots, X_N$ are independently normally distributed with mean m and variance σ^2, then the sample variance s^2 has $N-1$ degrees of freedom and has mean σ^2 and approximate variance $2(\sigma^2)^2/(N-1)$, or, still more approximately, $2(s^2)^2/(N-1)$. In the present case, then, the theoretical variance of U_i for an original chromosome is $2U_i^2/(6-1)=2U_i^2/5$, while the variance of U_{ij} for a cross is $2U_{ij}^2/(10-1)$. Now $V_{ij}=U_{ij}-B_{ij}$ has variance approximately $2U^2{}_{ij}/9$ + Var (B_{ij}), since B_{ij} depends only on M_{ij} and can be considered statistically independent of V_{ij}. Further, since B_{ij} is estimated from 1000 flies, Var (B_{ij}) is negligible compared to $2U_i^2/9$, and we have the variance of V_{ij} estimated by $2U_{ij}^2/9$. Finally, a suitable estimate of the variance of the V_{ij} based solely on variability within crosses is $2\overline{U}^2/9$, where $\overline{U}$ is the mean for the set of crosses in question. This estimate is entered as the "within" mean square and used in calculating the nonadditive between crosses F ratio and component of variance. Its use for the F ratio is not strictly correct, since $2\overline{U}^2/9$ is not distributed as a mean square, but it is based on so many more degrees of freedom than the numerator that the distribution of F is not seriously affected. Similar considerations apply to the original chromosomes, where $2\overline{U}^2/5$ is entered as the total residual mean square. For the crosses, a total residual mean square has been entered as $2\overline{U}^2{}_{orig}/9$, and used to obtain an estimated component of variance within crosses. These last entries serve no useful purpose except convenience in comparison; they give no information not already contained in the corresponding components for the means.

Nonparametric tests

It has been pointed out above that the F test requires the assumption of normality, although it is not very sensitive to moderate departures from this assumption. Even if the basic data, X_{ijk}, are normally distributed, the variances calculated from them, the U_{ij}, will not be normally distributed. However, the more degrees of freedom there are for an estimated sample variance the more nearly it is normally distributed. In the present case for the crosses the U's have nine degrees of freedom, and there is some evidence that this is sufficient to allow for the use of the analysis of variance. Since the binomial component has only a small variance, the V_{ij} are essentially distributed as the U_{ij} and we may use the analysis of variance on the V's without too much worry. Nevertheless, it might be preferable to use a test that did not require the assumption of normality. In the case of the V_{ij} such a test is available only for testing the additive effect. For the interpopulational crosses the test of FRIEDMAN (1937) also described in KENDALL (1948) may be used, while a modification of this test can be used for the diallel analysis of variance. These tests are based on ranks, and somewhat greater sensitivity can be obtained if these ranks are transformed into the equivalent normal deviate by the use of Table 20 in FISHER and YATES (1938). The F values for the

additive effect for the variances in Table 5 of each of the first three papers of this series were obtained by these nonparametric methods.

The interactions of interactions

The analysis of variance of variances was undertaken to investigate the question of whether σ_w^2 was constant or whether different w_{ijk}'s had different variances depending on the particular cross involved. Similarly, one can ask whether σ_d^2 is constant or whether the variance of d_{ij} depends on i and j (but not on the specific combination of the two); i.e., $Var\ (d_{ij}) = l_i + q_j$, say. In other words we may ask whether certain chromosomes have a tendency to react erratically, with a large nonadditive component, whereas other chromosomes are more consistent and the viability of chromosomes derived from crosses in which they take part may be predictable. The method used for testing this will be described more fully elsewhere. Here it will suffice to say that the individual d_{ij} were estimated by the difference between the mean viability for a particular cross and that predicted from the grand mean and the additive effects of the chromosomes involved, and that a nonparametric analysis of variance was done on the absolute values of these estimated interactions. The results are given in Table 5 of each of the first three papers in the lines labelled int. of int., for interaction of interactions.

Confidence limits for the components of variance

Confidence limits for a single mean square may easily be obtained by the use of a table of chi-square, and this method is explained, for example, in DIXON and MASSEY (1957). The genetic components of variance for the means and the components of variance between crosses for the variances are estimated by a difference of two mean squares. Confidence limits for these have been obtained by the use of the second approximation of BULMER (1957), with slight modification for the case of the component of variance within crosses for the means. For the means, confidence limits for the total residual component are obtained from the confidence limits for $\overline{U}$ for the original chromosomes with a slight modification. Confidence limits for the binomial coefficient are obtained from confidence limits for the grand mean for the cross. Finally the confidence limits for the real residual variance are obtained by subtracting the binomial estimated component from the limits for the total residual component. It should be noted that the lower limit for the total residual component is never allowed to be less than the lower limit for the binomial component, since the total residual variance is the sum of the binomial and real residual components.

Unlike the F test for the existence of the various components of variance, the method used for obtaining condence limits for these components is extremely sensitive to departures from normality in the data; consequently the limits as given can only be considered as very rough approximations giving the order of magnitude of our uncertainty in knowledge of the components. Whenever the F test in Table 5 of any of the papers showed that a certain component of variance was not significantly different from zero at the 2½ percent level of significance,

the corresponding lower 95 percent confidence limit for that component was set equal to zero in Table 6. On the other hand in one case for *Drosophila persimilis* the lower confidence limit of zero was obtained although a nonparametric F test showed the existence of a significant component. Since the nonparametric F test cannot be used to estimate components of variance, all that could be done was to indicate in the text that there was probably a significant component but that it could not be estimated.

The meaning of significance tests

When we say that a certain effect is significantly different from zero at the five percent level, we mean that if there were no true effect then we would obtain as large an estimated effect in less than five percent of all repetitions of the experiment. Similarly a standard error attached to an estimate is a measure of the variability of that estimate in repeated experiments on the same material. However, in many cases, the meaning of the words "repeated experiments" are by no means clear. For example, in the case of the present material, we can conceive of at least five kinds of repetitions of the experiment. (1) We can use the same chromosomes and crosses, repeated at the same time the original experiment was done. (2) We can repeat the experiment using the same chromosomes and crosses but at a somewhat later time, when the genetic contents of the chromosomes may have changed somewhat because of mutation, and experimental conditions will certainly be somewhat different. (3) We can repeat the experiment with other chromosomes taken at random from the same population. (4) We can repeat the experiment using other populations of the same species. (5) We can repeat the experiment using different species.

Each of these types of repetition involves all the sources of variation present in the previous kinds of repetition plus some additional ones, and consequently the further along we are in the series the greater the amount of variability that would be observed.

Since for each original chromosome six simultaneous replicates were tested, and for each cross ten derived chromosomes were tested simultaneously, we have available a repetition of type (1). The most important problem in the present papers is the comparison of the variability released by recombination within crosses with the variability between replicate cultures for the same original chromosomes. Here repetition of type (1) is appropriate and it is this repetition that furnishes the error estimates used in the analyses of variance and in the confidence intervals for the variance components. Since different crosses were tested at various times over a period of a year or more, and the original chromosomes were tested at still another time, a comparison of the variability between crosses with that within crosses should properly involve repetition of type (2). No repetition of this type is available, but the additional variability should be rather small, and thus the use of type (1) repetition should be satisfactory. It is true that for one locality, Texas, in *Drosophila pseudoobscura*, the original chromosomes were tested at two different times. However, these times were even

further apart and the conditions of the tests were somewhat different, so that this would give us an overestimate of the amount of additional variability in repetition of type (2).

If it is desired to make comparisons between the two localities for a given species still further problems arise. If we are simply interested in whether there are real differences between the two particular groups of chromosomes studied, then the proper error is obtained from the mean square within crosses. However, if we are really interested in whether there are differences between the localities *per se*, then we are interested in repetition of type (3); that is, the question is what would happen if we obtained new samples from these two localities. In this case the error must be obtained from the mean square between crosses, which is considerably bigger, and furthermore is derived from fewer degrees of freedom and consequently is less likely to yield significant differences. A further complication here is that the chromosomes used are not a random sample from the population in question, but were selected to have moderately high viability when homozygous. Consequently the conclusions are only valid for the class of randomly selected chromosomes of high viability. Finally, in comparing the behavior of different species, a further problem of differences between localities arises. If we can assume that all localities are the same, which is very doubtful, then replication of type (3) is proper and comparisons between species are made in precisely the same way as comparisons between localities for the same species. If, however, we make the more realistic assumption that there are differences between different localities for the same species, then repetition of type (4) is in order; that is we must compare the results from a random sample of localities for one species with the results from a random selection of localities for the second species. As, in the present case, we have only two localities for each species, it is very difficult to prove any differences between species.

The above, while rigorously correct, takes, perhaps, a somewhat pessimistic view of the matter. It is not always necessary to have results that are statistically significant at the five percent level. In some cases it may be enough if they are significant at the 50 percent level; that is, there would be only an even chance of observing such a difference if in fact no true difference existed. Furthermore, one can always make estimates of various effects and various differences, and it is not necessary to assume that such estimates will always differ from their true values by, for example, two standard deviation units. In fact most of the time they will differ by much less. Thus with the present data it would seem reasonable, for example, to say that there was a difference in the amount of variability released between Pirassununga and Rio, for *Drosophila prosaltans*, and also that there was a difference between the amount of variability released in *Drosophila pseudoobscura* and *Drosophila persimilis*, even though in neither case can the difference be rigorously proved statistically. In this connection it should be realized that even if perfectly correct statistical methods yield a result that is statistically significant at the one percent level, it is still possible that no effect actually exists and that this is just one of the one percent of the cases in which an

abnormally large observed effect is found. Thus, the difference between effects that are proved statistically in a rigorous fashion and those that are only shown in a somewhat looser way is not necessarily a hard and fast distinction.

It is evident from the above that a given single estimate, such as, for example, a mean viability, might have four different standard errors depending on the use to which the standard error was to be put, and attaching a single standard error could prove quite misleading. Accordingly, no standard error has been attached to such estimates, with the exception of a few cases where for some reason it was felt desirable to do so.

One other question of significance that arises is that of correlations. All correlations in these papers are based on ten chromosomes. If we are interested only in the correlation between these particular chromosomes, most of the coefficients observed are significant. If, however, we wish to make inferences about what would happen with ten other chromosomes from the same population, then the observed correlation must be less than -0.63 or greater than $+0.63$ to be significant at the five percent level. Evidently ten observations are too few to give much information about a correlation. Nevertheless the correlations are included, since the presence of many small ones and a number of negative ones, shows that in general there is little consistency in the contribution of a given chromosome under different conditions.

SUMMARY

This paper discusses the statistical methods used in earlier papers of this series to analyze data on *Drosophila pseudoobscura* (SPASSKY *et al.* 1958), *D. persimilis* (SPIESS 1959), and *D. prosaltans* (DOBZHANSKY *et al.* 1959). The use of diallel analysis of variance and two-way analysis of variance, combined with a one-way analysis of variance on control data, to test hypotheses about various components of variance, and to estimate these components and obtain approximate confidence intervals for them is explained. The definitions of the various components in terms of the mathematical model used and their genetic interpretation are given. This is done for components of variance of variances as well as for the more usual components of variance of means. Certain difficulties inherent in the interpretation of significance tests, confidence limits, and standard errors for complex experiments such as the present are discussed.

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STUDIES ON A SERIES OF TRYPTOPHAN-INDEPENDENT STRAINS DERIVED FROM A TRYPTOPHAN-REQUIRING MUTANT OF ESCHERICHIA COLI[1]

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STRAINS which approach wild type in appearance or behavior can arise from mutant strains as a result of either reversion (mutation at the original mutant locus) or suppression (mutation at some other genetic locus). In microorganisms both reversions (HOGNESS and MITCHELL 1954) and some suppressor mutations (YANOFSKY 1952; PARKS and DOUGLAS 1957) are known to act by restoring the specific enzyme activity which was lacking in the original mutants examined. Although it might be expected that reversion would always restore a mutant locus to its wild type form, recent studies with Neurospora indicate that this may not always be true. Both GILES (1957) and FINCHAM (1957) have reported cases in which reversion restored somewhat less than the wild type level of specific enzymes.

The purpose of the study reported in this paper was to examine the characteristics of the different types of strains which were obtained by mutation of a tryptophan- requiring mutant of *Escherichia coli* to tryptophan independence.

Terminology

The term "phenotypic revertant" is used to describe any tryptophan-independent (T^+) strain, regardless of genetic type, derived from the tryptophan- requiring mutant td_2. The term "reversion" or "revertant" will apply only to strains in which the restorative mutation appears to have occurred at the same locus as the original mutation.

MATERIALS AND METHODS

Escherichia coli strains: The tryptophan auxotroph from which the tryptophan-independent strains employed in this study were derived is designated td_2. This mutant was obtained from wild type strain K-12 by using ultraviolet light as the mutagenic agent, followed by penicillin selection. Nutritional and enzymatic studies with this auxotroph have shown that it will not respond to indole or anthranilic acid and specifically lacks tryptophan synthetase activity (the enzyme catalyzing the coupling of indole and serine to form tryptophan). When grown in liquid minimal medium with low tryptophan supplements (2–10 μg

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/ml), this strain accumulates large amounts of indole in its culture medium (YANOFSKY 1957).

In some experiments with strain td_2 it was desirable to have an unlinked marker present. For this purpose a histidine-requiring td_2 stock (td_2H^-) was prepared from an H^- cystine-dependent strain by transduction with bacteriophage grown on strain td_2. Since the cystine marker is closely linked to td_2, a large percentage of the cystine independent colonies obtained were td_2H^-. A stock bearing the same cystine marker (cys^-td^+) was used in all crosses and transductions.

Media: L-broth was used when a nutrient medium containing tryptophan was required (LENNOX, 1955), except in crosses, for which Difco Penassay broth was employed. The minimal salts medium of VOGEL and BONNER (1956) with 0.2 percent added glucose was used throughout and was supplemented with L-tryptophan when required. A supplement of 10 μg L-tryptophan/ml. permitted full growth of td_2. A supplement of 30 μg L-histidine/ml. was used when necessary.

Spontaneous reversion experiments: A 24–48 hour-old single colony isolated from strain td_2 was suspended in saline and the turbidity of the suspension determined. A series of L-broth tubes (10 ml/tube) were then inoculated so that each tube contained 10^4 bacteria/ml. At this level of dilution, tryptophan-independent cells would not be expected to be present. To verify this in each case, aliquots of the original suspension (10^5 cells) were plated on plates containing minimal agar plus 0.01 μg tryptophan/ml. The broth cultures were grown overnight with shaking and in the morning contained approximately 5×10^9 bacteria/ml. Saline dilutions were made from each L-broth tube and aliquots containing 10^7 cells were plated on plates containing minimal agar with the low level tryptophan supplement (0.01 μg/ml)[4].

Experimental and control plates were incubated for eight days at 37°. Although some tryptophan-independent colonies appear as early as one or two days after plating, the number of such colonies reaches a maximum after about eight days.

Reversion experiments with ultraviolet light: Strain td_2 was grown overnight in L-broth, and in the morning the culture was diluted to 10^8 bacteria/ml in a fresh tube of L-broth. This tube was incubated with shaking until a population of 5×10^8 cells/ml was obtained, and then this second culture was diluted to 10^7 bacteria/ml in minimal medium without glucose. Ten ml of this suspension were exposed to ultraviolet light at a distance of 40 cm. for ten seconds. With strain td_2 this exposure gives approximately 80–90 percent kill. Samples containing 10^7 cells were plated at zero time and after ten second exposure (10–20 percent viable) on minimal agar supplemented with 0.01 μg tryptophan/ml. The plates were incubated for eight days at 37°.

Routine handling of tryptophan-independent colonies: Colonies of widely varying sizes appeared after incubation of the experimental plates for eight days. These colonies were picked and streaked on minimal agar to obtain single colony

[4] This low tryptophan supplement will not support visible growth of td_2 but about ten times more ultraviolet-induced phenotypic revertants are recovered with this supplement than with minimal medium alone.

isolates before further tests were performed. Following this initial purification the T^+ strains were tested for indole accumulation and were plated to obtain an accurate determination of colony size.

Accumulation studies: Tubes containing minimal medium with 0.2 percent glucose or 0.2 percent glycerol were inoculated with the T^+ strains to be tested. These cultures were incubated with shaking for 40–48 hours at 37°. One part of the culture filtrate was mixed directly with two parts 95 percent ethyl alcohol and one part Ehrlich's reagent as a test for indole accumulation. Ehrlich's reagent gives a bright red color in the presence of trace amounts of indole.

Colony size studies: When isolates of different phenotypic revertants were first examined it was apparent that there was a wide range of individual growth rates. Accurate measurement of log phase generation time of a large number of these T^+ strains seemed impractical; consequently, colony size measurements were performed under controlled conditions. It was felt that such measurements would give a rough indication of the relative growth rate of any one mutant strain and would permit comparisons of the growth rates of the phenotypic revertants and of wild type. The log phase growth rate of selected T^+ strains was examined more carefully as described in the next section.

Single colony isolates from each T^+ strain were grown for 16 hours at 37° in nutrient medium. In this length of time each culture reached the stationary phase of growth and the cells were present at a concentration of about 5×10^9/ml. These cultures were diluted in saline and 10 to 30 cells were plated on minimal agar. The plates were incubated for 96 hours at 37°. At this time, the colonies were measured under a binocular microscope equipped with an ocular micrometer. The "average colony size" was obtained by taking the numerical average of the diameters of all the colonies on the plate. Colonies derived from some T^+ strains were quite uniform in size and showed a variation of only 0.1 mm or less between colonies. However, some strains consistently threw off fast-growing (large) colonies. These strains were usually the slowest growing isolates. In such cases, "average colony size" was not very meaningful and these strains were used in only a few of the experiments. In cases where it was desirable to use strains of this type there is an explanatory notation.

Experimental variation between duplicate plates in the same experiment and between experiments was determined for one T^+ strain having a relatively stable colony size. The variation observed was less than ten percent.

Growth curves: Log phase growth rates on liquid minimal medium were determined for selected T^+ strains. Erlenmeyer flasks (125 ml) equipped with sidearm colorimeter tubes were used in these studies. These flasks, containing 10 ml of medium, were inoculated to give an initial concentration of 10^8 bacteria/ml and were incubated with shaking in a 37° water bath. The turbidity of the cultures was measured with a Klett-Summerson photoelectric colorimeter (660 filter) at 30 minute intervals throughout lag phase and log phase.

Studies of enzymatic activity: Strains selected for enzyme analysis were grown in one liter volumes of minimal medium. Log phase cells were used as the starting

inocula. Each culture was incubated with shaking at 37° and its growth was followed closely. When the turbidity indicated that the culture had reached a population of 5×10^8 to 10^9 cells/ml an aliquot was removed for the determination of colony size; the remaining cells were harvested by centrifugation. The cells were washed once with saline and resuspended in 0.1M phosphate buffer (pH 7.8). These suspensions were then disrupted in a 9 kc Raytheon sonic oscillator and the cellular debris removed by centrifugation for 30 minutes at 15,000 g in an SS-1 Servall centrifuge. The supernatant solutions were used as crude enzyme preparations and were stored at −15°C.

Tryptophan synthetase activity was measured by the method of YANOFSKY (1957). One unit of tryptophan synthetase is that amount of enzyme which will convert 0.1 uM of indole to tryptophan when incubated for 30 minutes at 37° in the presence of indole, serine and pyridoxal phosphate. The protein content of the extracts was determined by the method of LOWRY *et al.* (1951). The tryptophan synthetase specific activity of an extract is expressed in units of enzyme activity per milligram of extracted protein.

Genetic studies: Suppressed mutants and true revertants were distinguished by using two genetic techniques, transduction and crossing. Transductions were performed with phage Plkc using the procedure described by LENNOX (1955). All crosses were performed in the following way: An F^+K-12 strain requiring cystine (*cys*$^-$ *td*$^+$) was grown overnight with shaking at 37° on Penassay broth plus added cysteine. Growth under these conditions presumably converts the F^+ character to an F^- phenocopy (LEDERBERG, CAVALLI and LEDERBERG 1952), and this strain was treated as the recipient. The tryptophan-independent strains obtained from the derived strain *td*$_2$*H*$^-$ were used as donors. These strains were grown overnight without aeration. In all crosses, 10^9 recipient cells and 10^8 donor cells were added to 2 ml of Penassay broth. This mixture was incubated with shaking for one hour at 37° and samples were diluted and plated on minimal agar enriched with 0.2 percent nutrient broth[5] and supplemented with 10 μg of L-tryptophan/ml.

The genetic constitution of the progeny from both transductions and crosses was tested by replica plating (LEDERBERG and LEDERBERG, 1952) followed by picking and streaking on appropriate media.

RESULTS

Frequency of appearance of indole accumulators among phenotypic revertants: Accumulation tests were performed on purified isolates chosen at random from the phenotypic revertants derived spontaneously and induced by ultraviolet light to determine whether these strains differed from wild type. The tests showed that most of the T^+ strains obtained were accumulators of indole, and that the percentage of nonaccumulators was smaller in the ultraviolet treated material (Table

[5] The *td*$_2$*H*$^-$ strain shows high frequency recombination for markers in the tryptophan region when the crossing mixture is plated on minimal medium enriched with 0.2 per cent nutrient broth (YANOFSKY and LENNOX, unpublished).

1). The work described in the Results section is entirely concerned with the behavior of the indole accumulators.

All of the ultraviolet-induced T^+ strains probably derive from independent mutational events, but under the conditions employed in the spontaneous reversion experiments some duplication of specific T^+ types could occur. Spontaneous T^+ strains must of course be independently derived if they appeared in separate experimental cultures or if they can be distinguished by characteristics of growth and enzyme content.

TABLE 1

Frequency of appearance of indole accumulators among tryptophan-independent mutants

	Ultraviolet		Spontaneous	
	Control (0 sec)	Experimental (10 sec)	Control	Experimental
Total cells plated (3 experiments)	1.2×10^8	4.6×10^7	3×10^5	2.8×10^8
Number of phenotypic revertants	22	363	0	45*
Frequency	$0.18/10^6$	$7.7/10^6$	0	$0.16/10^6$
Percent indole accumulators in random sample		90.9		54.0

* Including at least 22 independent events since this number of different experimental cultures were examined.

Variation in colony size among ultraviolet-induced and spontaneous phenotypic revertants: The indole accumulating strains employed in the colony size studies form an unselected sample. Twenty-nine spontaneous T^+ strains and 59 ultraviolet-induced T^+ strains were examined and the results are plotted in Figures 1a and 1b. These figures show that different patterns are obtained with the spontaneous and ultraviolet-induced populations. The spontaneous phenotypic revertants have average colony diameters which appear to be distributed randomly over a wide size range, 0.5 to 4.5 mm. Although a few of the ultraviolet-induced T^+ strains have colony diameters of 2.0 to 3.7 mm, most fall in the 0.8 to 1.7 mm class. There is probably another class at 0.5 mm.

This discrepancy between the two populations might possibly be explained by selection against slow growing spontaneous phenotypic revertants. In spontaneous reversion experiments, a phenotypic revertant must be able to grow and survive in a large population of td_s cells which are growing rapidly on the nutrient medium supplied. This is not the case in the experiments employing ultraviolet light. In these, the mutations presumably occur at or soon after the time of plating on minimal agar and there is, therefore, no possibility of competitive growth on a nutrient medium. If there is selection against slower growing T^+ strains in spontaneous mutation experiments the frequency of their appearance in plating experiments will not represent the actual frequency of this type of mutation. Experiments performed to examine this point did not reveal any obvious selection against slow growing phenotypic revertants on the nutrient medium employed.

The correlation of log phase generation time with colony size: The colony size differences observed in the experiments previously described might not reflect differences in growth rate but could be due to differences in the length of the lag phase. To examine whether colony size is correlated with log phase generation time, the log phase generation time of five spontaneous phenotypic revertants

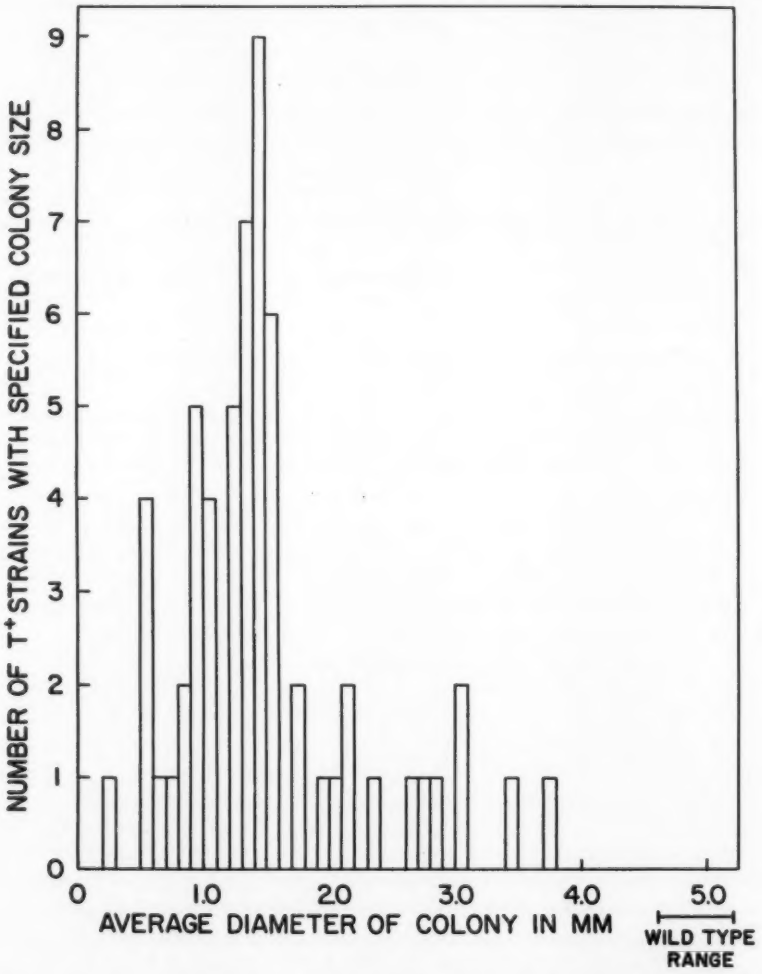


FIGURE 1a.—Average colony sizes in an unselected sample of indole accumulating ultraviolet-induced phenotypic revertants.

was determined and the values obtained plotted versus the colony sizes of the same strains (see Figure 2). It is clear from the figure that colony size appears to be a true reflection of the growth rate of these strains.

Genetic studies: Genetic studies were performed on a large number of indole-accumulating T^+ strains to determine whether the mutations conferring tryptophan-independence had occurred at the td_2 locus or at some suppressor locus.

Transductions were carried out in the following way. Phage was grown on the T^+ donor strains and the lysates obtained were mixed with cells of a cys^- td^+ recipient. Aliquots were plated on minimal agar supplemented with 10 μg tryp-

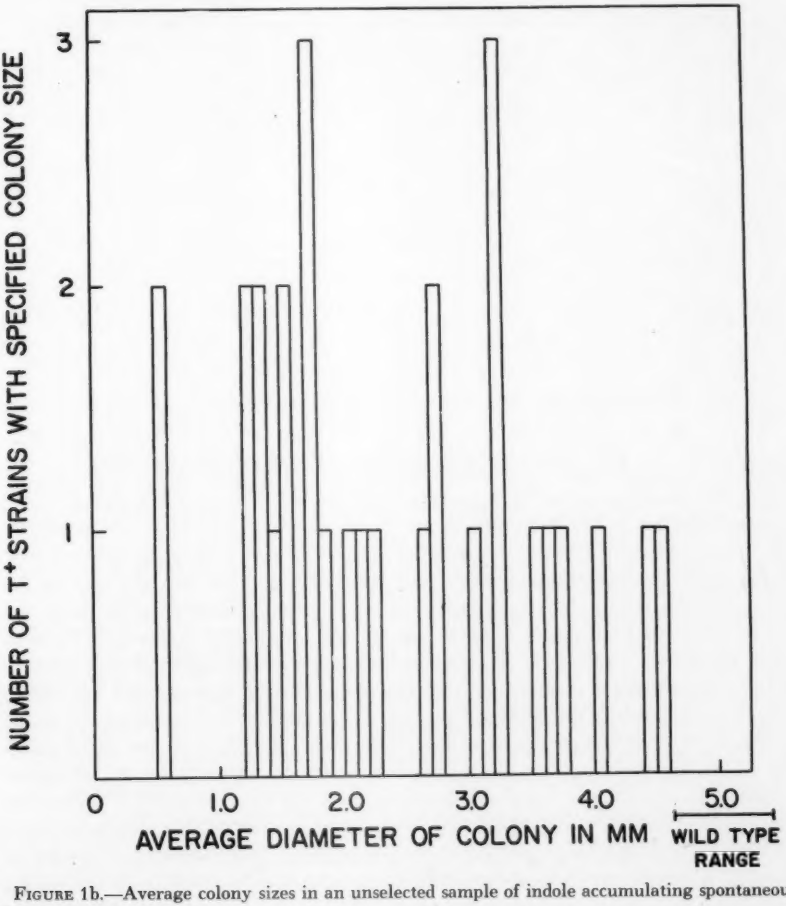


FIGURE 1b.—Average colony sizes in an unselected sample of indole accumulating spontaneous phenotypic revertants.

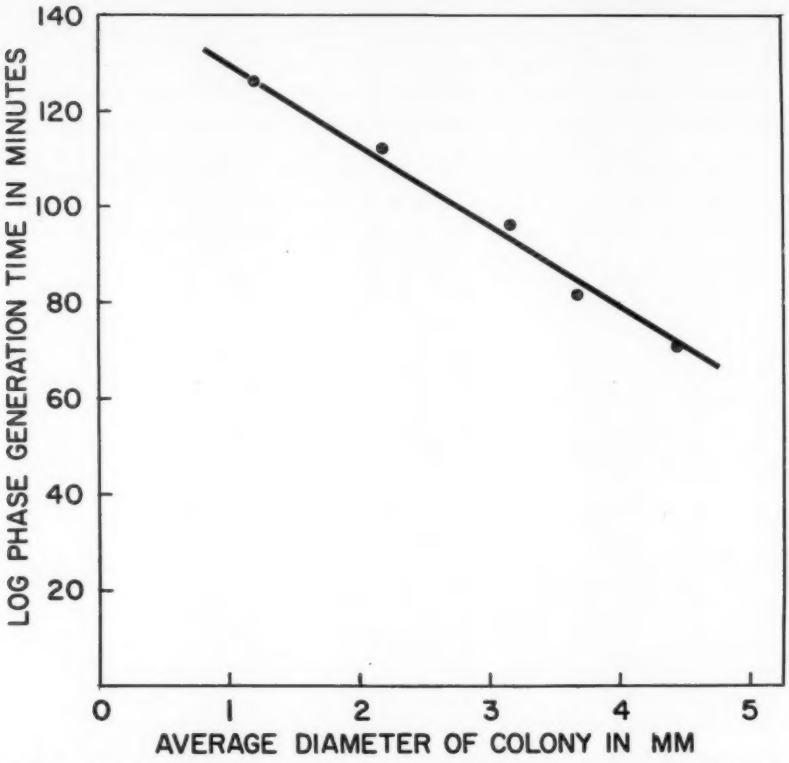


FIGURE 2.—Correlation of log phase generation time with colony size in spontaneous T+ strains selected for uniformity of colony size.

tophan/ml. thereby making the *cys*+ marker selective and the tryptophan marker nonselective. The transduced colonies were replicated to minimal agar and, if tryptophan requirers were present, the number of T+ and T− colonies were recorded. When they appeared, some tryptophan-requiring colonies were picked from each transduction and their nutritional requirement verified by streaking on appropriate media.

Since the *cys*− marker used in this experiment is linked to the td_2 locus and the *cys*+ marker of the donor strain was selected, some of the transduced colonies should carry the donor form of the *td* locus. If the donor strain carried td_2 unchanged, plus an unlinked suppressor gene, some of the transduced colonies should be tryptophan-dependent. If the suppressor gene were completely unlinked, approximately 36 percent of the *cys*+ colonies should require tryptophan (YANOFSKY and LENNOX, unpublished). This is the value obtained in control

transductions of td_2 into the *cys*⁻ strain. If the suppressor gene carried by the donor strain showed some linkage to the td_2 locus, the number of tryptophan-dependent colonies would be reduced. On the other hand, if the tryptophan-independent donor strain were derived from a mutation at the td_2 locus, tryptophan-requiring colonies would not be expected.

Genetic data obtained in transductions with selected ultraviolet induced T⁺ strains are presented in Table 2. Eight of the eleven phenotypic revertants tested

TABLE 2

Genetic data on selected ultraviolet-induced phenotypic revertants

Colony size	Genetic test	T⁻/colonies tested	Percent tryptophan requirers
2.7	Transduction	0/152	0
2.6	Transduction	0/123	0
2.1	Transduction	0/321	0
1.3	Transduction	73/193	37.8
1.3	Transduction	12/59	20.3
1.3	Transduction	5/44	11.3
1.1	Transduction	105/304	34.6
0.9	Transduction	16/91	17.7
0.5	Transduction	56/165	33.9
0.5	Transduction	35/121	28.9
0.2	Transduction	10/97	10.3
	Control transduction ($td_2 \rightarrow cys^-$)	72/215	33.5

T⁻ = colonies requiring tryptophan.

proved to carry suppressors, while three appeared to represent true reversions of the td_2 locus.

Genetic data on the spontaneous phenotypic revertants were obtained by crossing as well as by transduction. In the crosses the recipient strain was also *cys*⁻ td^+. Spontaneous T⁺H^- strains derived from strain td_2H^- were used as donors. After crossing, aliquots of dilutions were plated on enriched minimal agar supplemented with tryptophan. Thus, H^+ *cys*⁺ progeny were selected and the tryptophan requirement was kept nonselective, as in the transduction experiments. Control crosses of *cys*⁻ by td_2H^- showed that when the *cys*⁻ gene was replaced by *cys*⁺ from the donor, the td_2 gene was included about 32 percent of the time.

The rationale of the cross is the same as that of the transduction. If the donor strain contains td_2 and an unlinked suppressor gene, td_2 will be recovered in about 32 percent of the *cys*⁺ progeny. If the donor strain carries a td_2 reversion, no tryptophan requirers will be recovered in the progeny of the cross.

In order to check a large number of phenotypic revertants, a mixed crossing technique was devised. In this procedure, instead of examining each phenotypic

revertant singly, a mixture of several was employed as donor material. In control experiments of this type (see Table 3) it was shown that in a mixture of four strains, if one suppressed mutant was present, tryptophan-requiring colonies were recovered.

The results of genetic tests on selected spontaneous phenotypic revertants are presented in Table 4. Three of the 26 strains examined yielded tryptophan requirers and therefore carry suppressor mutations while the remaining 23 are true reversions. The results of tests on a random sample of spontaneous phenotypic revertants unselected for colony size are presented in Table 5. In this case all T^+ strains tested proved to be true reversions.

Correlation of genetic type with growth rate as determined by colony size: Most of the genetic studies were performed with spontaneous and ultraviolet-induced mutants selected on the basis of diversity of colony size. The three partial reversions obtained in the ultraviolet mutation experiments all have colony sizes greater than 2.0 mm, while all of the ultraviolet-induced phenotypic revertants with colony sizes of 1.3 mm or smaller proved to carry suppressor genes. Genetic tests with the selected spontaneous phenotypic revertants showed that

TABLE 3

Detection of suppressed mutants by a mixed-crossing technique

Crosses	T^- /colonies tested	Percent tryptophan requirers
Control:		
cys$^-$ × *td*$_2$*H*$^-$	21/68	30.9
Control:		
cys$^-$ × mixture: *td*$_2$*H*$^-$, S-3*H*, S-14*H*, S-28*H* (equal parts)	13/156	8.3
Mixed cross of unknowns which do not segregate td$_2$*:*		
cys$^-$ × mixture: S-13*H*$^-$, S-33*H*$^-$, S-32*H*$^-$, S-18*H*$^-$ (equal parts)	0/156	0
Mixed cross of unknowns which segregate td$_2$*:*		
cys$^-$ × mixture: S-1*H*$^-$, S-25*H*$^-$, S-19*H*$^-$ (equal parts)	18/161	11.2
Subsequent single crosses to test S-1*H*$^-$, S-25*H*$^-$, S-19*H*$^-$ *individually:*		
cys$^-$ × S-1*H*$^-$	31/80	38.7
S-19*H*$^-$	34/68	50.0
S-25*H*	0/46	0

T^- = colonies requiring tryptophan.

all but three of the mutant strains tested were actually true reversions. These three carried suppressor genes and they produce colonies with a diameter of 1.7 or 1.8 mm (Figure 3). It therefore appears that suppressed strains are characteristically slow growers (yielding colonies with diameters less than 2.0 mm) while the true reversions form colonies varying in size from 0.5 mm to 4.5 mm.

Maintenance of mutant characteristics through transduction: Although the particular characteristics of indole accumulation and slow growth were maintained by each mutant in serial transfer, it was desirable to determine whether these same characteristics were maintained through transduction.

In several tests with suppressed mutants, it was shown that the suppressor gene

TABLE 4

Genetic data on selected spontaneous phenotypic revertants

Colony size	Genetic test	T⁻/colonies tested	Percent tryptophan requirers
4.5, 4.4	Mixed Cross	0/141	0
4.0, 4.0	Mixed Cross		
3.7	Transduction	0/248	0
3.2	Transduction	0/36	0
3.2, 2.7	Mixed Cross	0/156	0
2.7, 2.6	Mixed Cross		
2.2	Transduction	0/49	0
2.1	Transduction	0/262	0
2.0	Transduction	0/120	0
1.8	Cross	69/146	47.3
1.8	Cross	0/587	0
1.7	Cross	155/301	51.5
1.7	Cross	74/124	59.7
1.5	Cross	0/100	0
1.5*, 1.4*, 1.3*	Mixed Cross	0/102	0
1.3	Cross	0/150	0
1.2	Cross	0/112	0
1.2	Transduction	0/87	0
0.56*	Mixed Cross	0/102	0
0.5	Transduction	0/61	0
	Control Transduction ($td_2 \rightarrow cys^-$)	168/493	34.1
	Control Cross ($cys^- \times td_2H^-$)	83/258	32.2

* These four strains were tested in the same mixed cross.
T⁻ = colonies requiring tryptophan.

TABLE 5

Genetic data on spontaneous phenotypic revertants unselected for colony size

No.	Colony size	Genetic test	T⁻/colonies tested	Percent tryptophan requirers
1, 2, 3	Unknown	Mixed Cross	0/264	0
4, 5, 6, 7	Unknown	Mixed Cross	0/332	0
8, 9, 10, 11	Unknown	Mixed Cross	0/296	0
12, 13, 14, 15	Unknown	Mixed Cross	0/286	0
16, 17				

T⁻ = colonies requiring tryptophan.

could be transduced into a td_z stock, yielding cultures which were indistinguishable from the original suppressed mutant. One experiment, for example, gave the following results when the control and transduction mixtures were plated on minimal agar: The td_z reversion control gave three tryptophan-independent colonies per 5×10^7 cells plated, transduction of td_z into td_z gave one colony per 5×10^7 plated, while transduction of the two suppressor strains into td_z gave 200 to 300 slow-growing colonies per 5×10^7 cells plated. In this experiment, twenty colonies were taken at random from each of the experimental plates and tested for indole accumulation. All of the colonies tested were indole accumulators and therefore the suppressor characteristics (slow growth on minimal agar and indole accumulation) were clearly transduced.

Although the frequency of mutation of the recipient strain to slow-growing indole accumulators was much lower than the transduction frequency of the suppressor gene, it was felt that studies on the inheritance of a particular growth rate through transduction should be performed on transduced mutants which could not possibly be mistakenly identified with new phenotypic revertants. When a true reversion is transduced into *cys*⁻ it is recovered in the *cys*⁺ progeny about 36 percent of the time. Four reversions (F, E, B, and H) were reisolated in this new genetic background (*cys*⁻ strain) and their growth rates compared with the original revertant strains. A new wild type strain was also isolated from the products of one of these transductions. This strain was compared with the wild type K-12 strain which had been used as a control in other growth rate

experiments. The value given for reversion E (before transduction) was obtained in a previous experiment.

It can be seen in Table 6 that, with the exception of strain H, the log phase generation time of the original strains and of the transduced strains agrees fairly

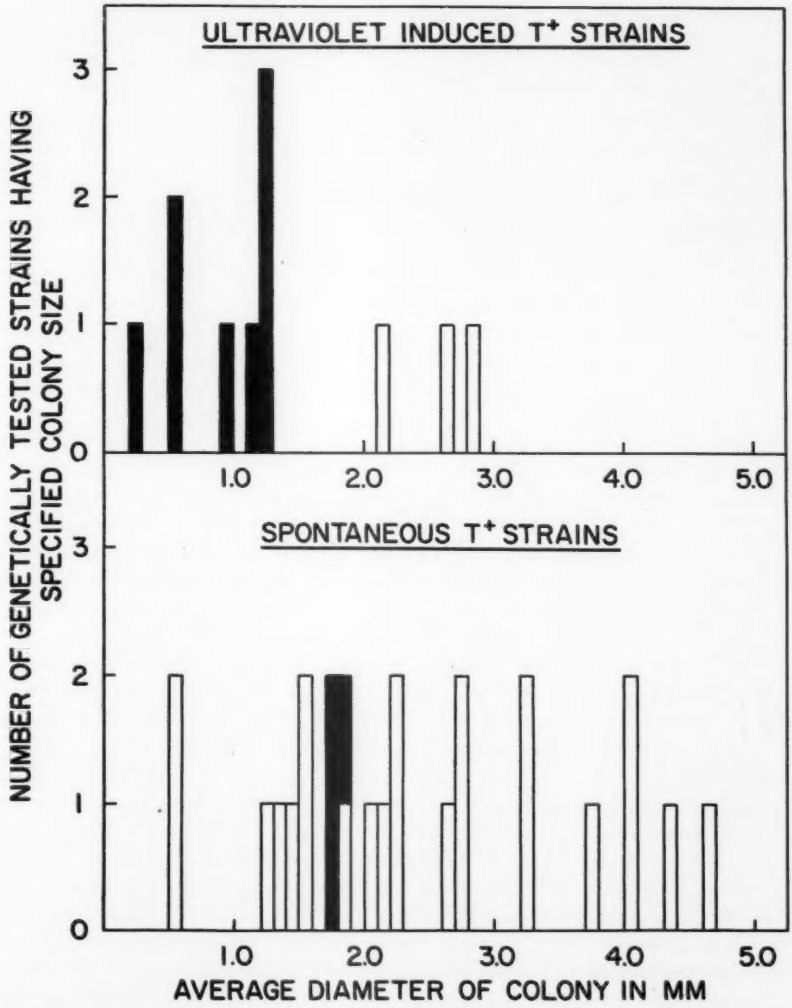


FIGURE 3.—Correlation of genetic type with average colony size. Black bars represent suppressed mutants and white bars, true revertants.

TABLE 6

Log phase generation time of true revertants before and after transduction into the cys- *strain*

Stocks examined	Generation time before transduction*	Generation time after transduction
Reversion F	84′	87′
E	93′	102′
B	150′	165′
H	210′	189′
Prototroph recovered from F — *cys-*		66′
Wild type K-12	60′	

* = on unsupplemented minimal medium.

well. The small differences which were observed were not much greater than those obtained with the wild type control. The one exception, strain H, grows about ten percent faster after transduction than before. Since this strain is variable with respect to colony size, it is possible that a faster growing colony was picked from the transduction plate. In spite of this exception, the data on the other three reversions suggest that the growth rates characteristic of reversions are maintained through transduction. *Thus it would appear that the factor or factors controlling growth rate are associated with the form of the td locus which was transferred by transduction.*

Tryptophan synthetase activity in extracts of selected suppressed strains and revertants: To determine whether tryptophan synthetase activity is correlated with growth rate, revertants representing seven different colony size classes and suppressed strains representing two different colony size classes were selected for enzyme studies. Tryptophan synthetase activity was detected in the extracts of each phenotypic revertant examined (Table 7). However, there was marked

TABLE 7

The tryptophan synthetase specific activity of various suppressed mutants and revertants

Strain	Average diameter of colony	Generation time	Tryptophan synthetase specific activity
	mm.	minutes	
suppressed strain A*	0.5	192	0.57, 0.71
suppressed strain B	0.9	126	0.7
suppressed strain C	1.3	117	0.63, 0.71
suppressed strain D	1.3	135	0.5, 0.72
reversion A*	0.56	114	0.4
reversion B	1.2	123	0.3, 0.28
reversion C	2.2	111	0.8
reversion D*	2.7	96	1.9, 2.9
reversion E	3.2	93	0.8
reversion F	3.7	81	1.1, 1.25
reversion G	4.4	72	0.23, 0.26
wild type	5.0	69	2.9, 3.0

* = Some faster growing colonies were evident on the control platings of these strains.

variation in the level of enzyme activity. When comparisons of activity of the extractable tryptophan synthetase are made between extracts of any phenotypic revertants, the reliability of the procedure employed must be considered in detail. As stated earlier, cultures were harvested for extraction when the concentration of cells was somewhere between 5×10^8/ml and 10^9/ml. One revertant was grown many times and repeatedly sampled at levels of growth corresponding to 5×10^8 cells/ml and 10^9 cells/ml. The variation between the activities of these extracts (roughly ten percent) is no more than the variation observed between extracts from cells grown at different times and sampled at the same turbidity. In all cases colony size controls were performed on the inoculum and on the cells at the time of harvesting. With the exception of reversions A and D (as noted in Table 7) no pronounced selection for faster growing types occurred.

The four selected suppressed strains have tryptophan synthetase specific activities only about 20–25 percent that of wild type K-12 (Table 7). Although differences in growth rate occur among the four suppressed stocks, this is certainly not reflected in the tryptophan synthetase activity of their extracts.

The seven selected revertants (Table 7) show much more variation in their specific activities—from ten percent of wild type activity to values approaching the wild type range. Enzyme content is not, however, strictly correlated with colony size. Reversion G grows well on minimal agar (average colony diameter, 4.4 mm) but has only ten percent of the wild type tryptophan synthetase activity. Conversely, reversion D grows more poorly (average colony diameter, 2.7 mm) but has high tryptophan synthetase specific activity. However, the control plates in the reversion D experiments showed evidence of the appearance of faster growers during the experiment. Colonies on these plates varied from 2.0 mm to 3.3 mm in diameter. If the colonies of 3.3 mm represented the fastest growers in the population, this strain still shows more tryptophan synthetase activity than would be expected in comparison with the other reversions.

If reversions D and G are omitted from the comparisons, there is a very rough positive correlation between growth rate and specific activity.

All of the extracts examined were frozen after sonic treatment and tryptophan synthetase assays were usually performed within one to two days after the preparation of the extract. On a few occasions, however, an extract was reassayed a week or more after the original determination had been made. In three of these cases (reversions B, F, and G) the enzymatic activity had dropped considerably. The tryptophan synthetase activity in extracts of wild type K-12 remains stable under these conditions for as long as six months.

Genetic examination of the site of mutation in reversions: The fact that many of the revertants examined differed considerably from wild type in growth rate, accumulation behavior, and enzyme content raised the possibility that the second mutation, although apparently occurring within the *td* locus, may have occurred at a site distinct from the site of the original mutation. If this were the case, it would be expected that the original mutant area could be reisolated by a rare crossover event within the *td* locus.

With this in mind, crosses similar to those previously described were performed on a larger scale. Four histidine-requiring td_2 reversions, A, D, G, and I, were crossed with cys^- td^+ and plated in the presence of tryptophan. The cys^+ recombinants obtained were tested for their tryptophan requirement (Table 8).

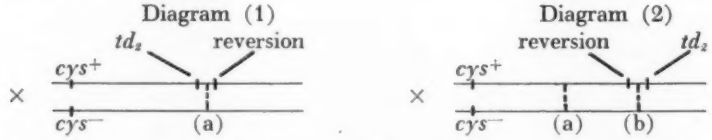
TABLE 8

Tests for a rare crossover event in several revertants

Reversion*	Genetic test	T⁻/colonies tested
A	Cross: cys⁻ × AH^-	0/421
D	Cross: cys⁻ × DH^-	0/860
G	Cross: cys⁻ × GH^-	0/1308
I	Cross: cys⁻ × IH^-	0/819

* = The four reversions tested have widely different characteristic colony sizes.
T⁻ = colonies requiring tryptophan.

In analyzing the results of these crosses, two alternative possibilities must be considered: first, the site of reversion may be to the right of the td_2 mutant area (see diagram 1), and second, the site of reversion may be between cys^- and the td_2 mutant area (see diagram 2).



If model (1) describes the situation, a rare single crossover at (a) would give a td_2 type in the cys^+ progeny. If model (2) is correct, a double crossover (a) (b) would be required to give a cys^+ td_2. However, in bacteria, it is probable that only fragments of the donor chromosomes participate in recombination. In this case a double crossover in (1) and a quadruple crossover in (2) would be required to give cys^+ td_2.

Large numbers of cys^+ progeny were tested and no T^- (cys^+ td_2) types were recovered. Since none were recovered, either the second mutation occurred at or extremely close to the site of the original mutation, or model (2) may represent the true situation in these particular strains.

DISCUSSION

Tryptophan-independent phenotypic revertants obtained from the tryptophan-requiring mutant td_2 arise by both suppressor mutation and reversion. More than half of the phenotypic revertants which are recovered accumulate indole even though these same strains are able to grow in the absence of exogenously supplied tryptophan. This is true for both suppressed strains and true revertants. Of particular interest is the fact that in many of the strains examined wild type physiological activity was not quantitatively restored by reversion at the original

mutant locus. The name *partial reversion* will be used to describe this situation. Furthermore, the partial reversion class has been shown to include a striking variety of revertants that can be distinguished by characteristic growth rates and levels of enzymatic activity.

Although, as expected, the number of phenotypic revertants increased after ultraviolet irradiation, the distribution of colony sizes was different from that observed in spontaneous mutation experiments. The spontaneous phenotypic revertants generally were of many different colony size classes with each of these classes including about the same number of strains. However, following ultraviolet treatment, proportionately more colonies less than 2.0 mm in diameter were obtained. Genetic studies have shown that these slower growing ultraviolet induced phenotypic revertants are suppressed mutants while the faster growing types are partial revertants. In the spontaneous mutation experiments, most of the T^+ strains obtained were partial revertants. The few suppressed mutants which were isolated produced colonies 1.7–1.8 mm. in diameter.

Considering these facts, it would seem that the ultraviolet treatment produces more suppressor mutations than true reversions. Although negative selection might explain the relative rarity of appearance of suppressed strains in the spontaneous mutation experiments, in a recovery experiment which was performed this was not the case. The explanation for the observed difference is not known.

The enzyme studies shows that tryptophan synthetase activity has been restored to some extent in both the suppressed mutants and the partial revertants. The strains chosen for these enzyme studies have widely different growth rates and it was hoped that, within each genetic group, there would be some correlation of growth rate with enzymatic activity. Such a correlation would be expected if tryptophan synthetase activity were the only factor limiting growth. The fact that all of these strains exhibit a growth response to added tryptophan demonstrates that the synthesis of tryptophan is one of the growth limiting factors. The four suppressed stocks which were examined showed no correlation between growth rate and enzyme content. Thus the full effect of suppressor mutations cannot be measured in terms of the amount of extractable enzyme present in crude preparations. With the partial reversions, there is some suggestion of a very rough correlation between growth rate and the amount of extractable enzyme activity; however, one of the partial reversions examined has much less tryptophan synthetase than would be expected and one has much more. Certainly for reversion D, which grows almost as well as wild type but has only ten percent of the wild type enzyme activity, it can be said that the physiological effect of the reverse mutation is not reflected in the quantity of extractable tryptophan synthetase which was detected.

In view of the poor correlation in some cases between extractable enzyme levels and growth rate and the preliminary observation that the tryptophan synthetase in some of the partial revertants may be relatively labile on storage, it might be postulated that all of these partial reversions represent qualitative changes in tryptophan synthetase, i.e., that mutation has restored the ability to

form tryptophan synthetase but the enzyme formed is not identical with the wild type enzyme. Similar observations of instability or of alteration of specific enzymes restored by reversions have been reported by GILES (1957) and by FINCHAM (1957) in studies with Neurospora.

It was mentioned previously that, in addition to the indole accumulating phenotypic revertants, nonaccumulators were also obtained. These were originally believed to represent complete reversion to wild type rather than partial reversion. Preliminary enzyme and growth studies, however, indicate that these nonaccumulators do not exhibit wild type characteristics although those strains which were examined were found to be true revertants by genetic tests.

In addition it should be emphasized that although the mutational behavior described in this paper is characteristic for strain td_2, an allele (td_4) of this mutant seems to behave quite differently. Preliminary investigations with td_4 suggest that it always reverts to a nonindole accumulating, fast-growing type.

Preliminary genetic tests were performed with the partial revertants of strain td_2 in an attempt to separate the sites of the original and second mutations. No evidence of separability was obtained. However, in view of the fact that many distinguishable partial revertants, each possessing tryptophan synthetase activity, are recovered from a single mutant, it would appear unlikely that all of the reversions occurred at the exact site of the original mutation. Rather, it is more probable that mutational changes at other sites within the same gene can result in the formation of tryptophan synthetase proteins with characteristically different turnover numbers.

SUMMARY

1. Tryptophan-independent (T^+) phenotypic revertants were obtained from td_2, a tryptophan-requiring mutant of *E. coli* strain K-12, both spontaneously and following ultraviolet treatment.

2. Genetic studies show that T^+ phenotypic revertants, either spontaneous or ultraviolet induced, arise both by suppressor mutation and reversion. Proportionately more suppressor mutations occur following ultraviolet treatment.

3. Growth rate studies demonstrate that suppressed mutants are characteristically slow growers, while true revertants have widely different growth rates only sometimes approaching the wild type level. Revertants of this type are called partial revertants.

4. The enzyme studies performed show that tryptophan synthetase activity has been restored to some extent in both suppressed mutants and partial revertants. However, studies of suppressed mutants and partial revertants with widely different growth rates show that in most cases enzymatic activity probably cannot be correlated with growth rate. Also, the extracted tryptophan synthetase of three partial revertants is more labile on storage than is the wild type enzyme. In view of these two latter observations it is postulated that in partial reversion the back mutation has restored the ability to form tryptophan synthetase but that the enzyme may not be qualitatively comparable to the wild type enzyme.

5. Preliminary large-scale genetic tests with four partial revertants have failed to separate the sites of the original and the second mutation. However, considering the fact that many distinguishably different partial revertants, all with tryptophan synthetase activity, are recovered from a single mutant it is proposed that some of the second mutational changes may have occurred at other sites within the original mutant gene.

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THE RELATION OF OXYGEN POSTTREATMENT AND HETEROCHROMATIN TO X-RAY-INDUCED CHROMOSOME ABERRATION FREQUENCIES IN CREPIS CAPILLARIS[1]

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SEVERAL studies have now demonstrated that postirradiation oxygen treatments can markedly increase the initial X-ray damage in barley seeds (ADAMS, NILAN AND GUNTHARDT 1955; NILAN 1955; ADAMS AND NILAN 1958; CALDECOTT *et al.* 1957; CURTIS, CALDECOTT AND KONZAK 1957). One of the more striking of these after effects is the marked increase in the frequencies of chromosome aberrations (ADAMS, NILAN AND GUNTHARDT 1955; ADAMS AND NILAN 1958).

The present investigation was undertaken to determine if the influence of oxygen after X-irradiation was random or differential in its action on the location of chromosome breaks in seeds. In other words, does oxygen increase chromosome aberrations in particular regions of the chromosomes?

Such a study required a detailed analysis of the chromosome aberrations that was not possible with barley. Therefore, *Crepis capillaris* ($2n = 6$) with its readily identified chromosome arms was chosen as the experimental organism.

In addition to providing data on the original objective, the study also disclosed some new information concerning chromosome breakage in Crepis. Here the increased chromosome rejoining in heterochromatic regions is of particular importance.

MATERIALS AND METHODS

The experiments were conducted with resting seeds of *Crepis capillaris* ($2n=6$). This species has only three pairs of chromosomes (A, C, and D) and each chromosome arm is readily identified. Chromosome A is the longest and its short arm is longer than the short arm of either of the other two chromosomes; C is the shortest and its short arm is medium in size; D is medium in length and its short arm is very small and has a tiny satellite attached. The average lengths of the chromosome arms at mitotic metaphase are as follows: Al 6.0 μ, As 1.8 μ; Cl 3.0 μ, Cs 1.0 μ; Dl 4.9 μ, Ds 0.5 μ. These measurements and a structural alteration in the D chromosome of the strain used in this investigation have been reported elsewhere (NILAN and SIRE 1958).

Samples of seeds at eight percent moisture obtained by storage over dry $CaCl_2$

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were X-irradiated at 1500r and 3500r in a single layer on a turntable which was centered 42 cm beneath a beryllium window of an AEG 50 Machlett X-ray tube. The tube was operated at 34 KV and 25 ma without filter. The high voltage supply produced full wave rectification smoothed out by a 0.2 microfarad condenser. The voltage was read on a meter having voltage resistance in series. Doses were given at the rate of 990r per minute at the surface of the seed as measured by a Victoreen condenser r meter with a nylon walled 250r chamber.

The oxygen gas for the postirradiation treatments was obtained from commercial cylinders (Industrial Air Products) and assayed as follows: 99.6 percent 0_2 + 0.4 percent N_2. The X-irradiated seeds were immediately placed in specially constructed chambers under 100 lbs pressure and stored for 42 days after the irradiation. During the posttreatment the gas was changed every three days. Samples of the seeds were analyzed cytologically immediately after, and 3, 15, 28, and 42 days after the irradiation. Samples of nonirradiated seeds were analyzed before storage and after 42 days of storage in oxygen.

Following the various treatments the seeds were germinated on filter papers in petri dishes. When the root-tip cells were undergoing their first mitotic divisions, the seeds were placed in .002 M 8-hydroxyquinoline for 1½–2 hours. Aceto-orcein squash preparations were made of the root tips, and the slides were made permanent with Euparal. This technique has been described in greater detail by TJIO and LEVAN (1950).

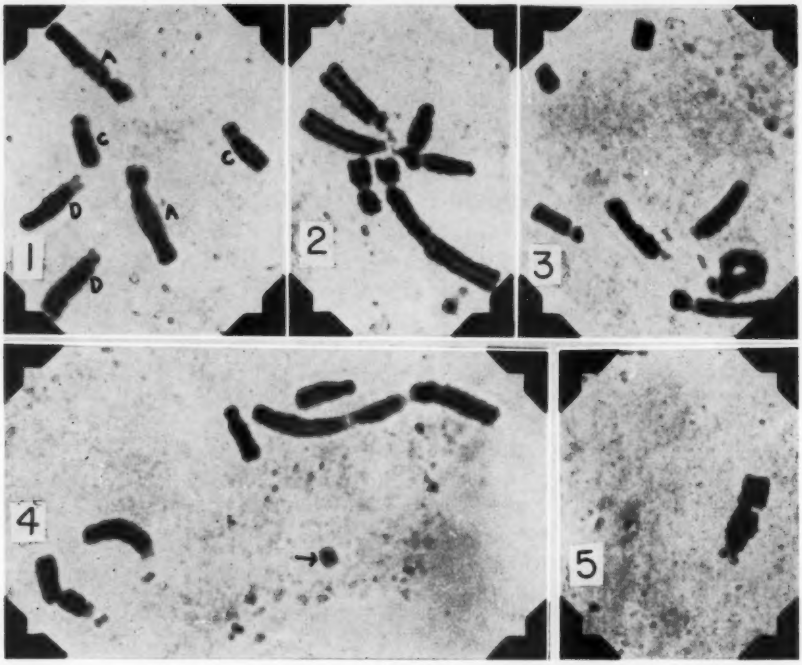
The cytological analysis consisted of recording the numbers of normal and disturbed cells, frequencies of dicentric chromosomes, and frequencies of isodiametric and rod acentric fragments at metaphase. Except for a few chromatid breaks, all of the aberrations were of the chromosome type.

Chromosome breaks which rejoined to form dicentric chromosomes were tabulated. These breaks were the easiest to identify and could be located with considerable accuracy. Translocations resulting from the rejoining of centric and acentric fragments were more difficult to identify. The total number of detectable breaks was determined by adding the dicentric breaks, other translocation breaks, and simple breaks (no rejoining). The majority of the simple breaks were identified and located from scoring rod fragments that had not restituted or rejoined and that were not associated with a translocation. This was a more reliable measure of simple breaks than scoring from isodiametric fragments. Nevertheless, these simple breaks could not be identified and located as easily as the dicentric breaks. Intrahomologous dicentrics and chromatid breaks were also recorded. The breaks in the various chromosome arms of many cells were located by drawing the chromosome aberrations resulting from the breaks. The normal chromosome complement of *Crepis capillaris* and some of the aberrations observed are shown in Figures 1 to 5.

RESULTS

Effects of oxygen

Chromosome aberration frequencies: Postirradiation treatment of 100 lbs pressure of oxygen increased the frequencies of abnormal cells, dicentric chromo-



FIGURES 1–5.—Photomicrographs of somatic metaphase chromosomes of *Crepis capillaris* showing X-ray induced aberrations. All figures are *ca.* × 2600.

FIGURE 1.—Chromosomes A, C, and D of a normal cell.

FIGURE 2.—Intrahomologous translocation involving the long arms of A; no fragments.

FIGURE 3.—Dicentric chromosome involving the long arm of A and the long arm of C; two pairs of rod fragments.

FIGURE 4.—Intrahomologous dicentric chromosome involving the short arms of the A chromosomes; one isodiametric fragment (arrow) and one pair of fragments just detached from the long arm of D.

FIGURE 5.—Chromatid break in the long arm of D.

somes and isodiametric and rod fragments (Table 1). During 42 days of postirradiation treatment aberration frequencies gradually increased. The isodiametric fragment frequencies increased faster than the rod fragment frequencies. Seeds X-rayed at 3500r and given the same posttreatments showed similar increases in abnormal cells, dicentrics, and fragments. Nonirradiated seeds stored for 42 days in oxygen showed considerably more aberrations than the control.

Proportions of breaks in the chromosome arms: Table 2 presents the total and dicentric breaks in each chromosome arm immediately following X-irradiation, and after postirradiation treatments of oxygen. Since X-ray dose did not alter

TABLE 1

Frequencies of abnormal cells, chromosome dicentrics, and rod and isodiametric fragments in X-irradiated (1,500r) seeds of Crepis capillaris *stored in oxygen at 100 lbs pressure from 0 to 42 days*

Treatment		Cells		Chromosome aberrations		
X-ray	Posttreat. (days)	Number	Abnormal percent	Dicent. /cell	Iso. frag. /cell	Rod frag. /cell
1500r	0	393	24.9	.09	.16	.23
1500r	3	193	50.3	.26	.55	.59
1500r	15	319	77.7	.32	1.75	1.07
1500r	28	250	82.8	.36	2.10	1.09
1500r	42	277	90.6	.52	3.41	1.89
0r	0	172	1.2	.006	.000	.000
0r	42	281	4.2	.018	.035	.035

TABLE 2

Proportion of total breaks and breaks leading to dicentrics in each chromosome arm of X-irradiated (1,500r and 3,500r) seeds of Crepis capillaris *stored in oxygen at 100 lbs pressure from 0 to 42 days*

O_2 post treatment	Breaks	Chromosome arms Al	As	Cl	Cs	Dl	Ds
None	Total no.	503	188	368	70	401	57
	Percent	31.7	11.9	23.2	4.4	25.3	3.6
	Dicent. no.	198	81	209	48	182	36
	Percent	26.3	10.7	27.7	6.4	24.1	4.8
3 days	Total no.	45	19	35	10	41	6
	Percent	28.8	12.2	22.4	6.4	26.3	3.8
	Dicent. no.	27	10	22	8	29	4
	Percent	27.0	10.0	22.0	8.0	29.0	4.0
15 days	Total no.	93	52	73	23	90	11
	Percent	27.2	15.2	21.3	6.7	26.3	3.2
	Dicent. no.	41	24	49	15	48	7
	Percent	22.3	13.0	26.6	8.2	26.1	3.8
28 days	Total no.	99	47	53	19	75	12
	Percent	32.5	15.4	17.4	6.2	24.6	3.9
	Dicent no.	54	24	35	10	47	10
	Percent	30.0	13.3	19.4	5.6	26.1	5.6
42 days	Total no.	121	59	95	39	102	17
	Percent	27.9	13.6	21.9	9.0	23.6	3.9
	Dicent. no.	70	42	73	30	63	12
	Percent	24.1	14.5	25.2	10.3	21.7	4.1
χ^2 on total breaks obtained					36.06		
χ^2 on total breaks expected (25 d.f.)					37.65 at 5% level 44.31 at 1% level		

the proportion of breaks in each chromosome arm the data from the 1500r and 3500r treatments were combined.

By inspection it can be seen that the oxygen treatments did not alter the proportion of these breaks in each arm. This conclusion is supported by the fact that none of the differences were significant according to a chi-square test of independence in a row × column heterogeneity table (SNEDECOR 1956).

Rejoining of chromosome breaks: The number of breaks that rejoined was determined in the following manner: The number of dicentrics in each treatment (Table 1) was scored with considerable reliability. However, translocations, other than dicentrics, were more difficult to score. If the two translocated segments were approximately the same length, it was impossible to know if an aberration was present. Thus, the total number of translocations (rejoined chromosomes) were considered to be double the number of dicentrics. It is assumed in this calculation that if two chromosomes break in a cell, chance determines which two ends come together to form the reconstituted chromosomes. If breaks occur in any two arms and rejoining takes places, the result can be either a dicentric chromosome plus one (Figure 4) or two (Figure 3) fragments *or* two translocated chromosomes with no fragments (Figure 2). According to LEWITSKY and SIZOVA (1935) the "X-ray translocations in Crepis are for the most part, if not all, reciprocal." The present study supports this conclusion since most dicentrics were accompanied by only one fragment. In addition, there was no evidence of preferential rejoining between certain arms of the chromosomes in any of the treatments applied.

The breaks that did not rejoin were computed from the fragment number. This number was halved since each pair of fragments scored was the result of one break. Moreover, the fragments remaining from dicentric formation were subtracted. The breaks that remained were those leading to fragments that did not rejoin or become restituted.

The data in Table 3 which were computed in the above manner reveal that X-irradiation without oxygen posttreatment produced 4.3 breaks that rejoined to one break that did not rejoin. However, after 15 days of postirradiation treatment

TABLE 3

Ratios of chromosome breaks that rejoined to simple breaks (breaks that did not restitute or rejoin) in X-irradiated (1,500r) seeds of Crepis capillaris *stored in oxygen at 100 lbs pressure from 0 to 42 days*

O_2 post treatment	Dicentric bridge no.	Breaks that rejoined	Fragment pairs	Simple breaks	Rejoined breaks / Simple breaks
None	34	136	75	41	4.3
3 days	50	200	110	60	3.3
9 days	51	204	165	104	2.0
15 days	102	408	450	348	1.2
28 days	90	360	401	311	1.15
42 days	145	580	734	589	1.0

in oxygen the ratio was reduced to 2:1 and after 42 days it was further reduced to 1:1.

Distribution of breaks in relation to chromosome length

If the chromosome breaks induced by X-rays are random, then the frequency of breaks in each chromosome arm should be directly related to the length of the arm. To ascertain the expected distribution of the breaks in *Crepis capillaris*, the length of each arm was converted to a percentage of the total length (17.2 microns at mitotic metaphase) of all the chromosomes. The expected number of breaks in each arm was determined by multiplying the percentage figure by the total number of breaks in all chromosome arms. Chi-square tests were conducted to determine if the differences between the number of observed breaks and expected breaks in each arm were significant.

The observed number of breaks leading to dicentrics was much greater than expected in the long arm of C and less than expected in the long arm of A (Table 4). On the other hand, there were more simple breaks than expected in the long

TABLE 4

Proportion of breaks leading to dicentrics and simple breaks in each chromosome arm of X-irradiated Crepis capillaris *seeds*

(Summary of 8 replications)

Type of break	Al	As	Cl	Cs	Dl	Ds
Breaks leading to dicentrics						
Observed	198	81	209	48	182	36
Expected on basis of length	259	78	130	43	212	22
χ^2	15.00**	.05	44.42**	.21	4.53*	6.50*
Simple breaks						
Observed	77	13	21	0	38	0
Expected on basis of length	52	16	26	9	42	4
χ^2	12.02**	.56	.96	9.0**	.38	4.0*

arm of A and less than expected in the short arm of C. These differences are significant at the one percent level. The proportions of breaks in the remaining arms were not significantly different from expected. This differential breakage in relation to chromosome length was not influenced by oxygen posttreatment.

Relation to heterochromatin

Location of chromosome breaks: After many chromosome breaks were observed, it became clear that a large majority of the breaks leading to dicentrics occurred in or near the heterochromatic regions of the chromosome arms. These regions can be determined in prophase chromosomes in root-tip cells treated with oxyquinoline (Tjio and Levan 1950). The entire short arm and the proximal

and the very distal end of the long arm of each chromosome are heterochromatic. Furthermore, the heterochromatic areas in the long arms are approximately equal in size. Similar areas of heterochromatin in the chromosomes of *Crepis capillaris* have been pictured earlier (KOSTOFF and ARUTIUNIAN 1938).

Two hundred dicentric breaks were analyzed, and the locations of the breaks were tabulated (Table 5). Sixty-five percent of the breaks were definitely lo-

TABLE 5

Frequency of X-ray induced breaks leading to dicentrics and simple breaks in euchromatic and heterochromatic regions of chromosomes of Crepis capillaris

	Heterochromatic region		Euchromatic region		Euchromatic or heterochromatic region	
	Number	Percent	Number	Percent	Number	Percent
Breaks leading to dicentrics	129	65	4	2	67	33
Simple breaks	24	24	62	62	14	14

cated in heterochromatin, 2 percent in euchromatin; the remaining 33 percent could have been in either area. However, most of these questionable dicentrics were more likely formed from breaks in heterochromatin for usually the dicentric was of the same length as one or the other of the donor chromosomes. In other words, one donor chromosome had broken near the centromere and the other had broken near the distal end of the long arm.

One hundred simple breaks were scored for their distribution on the chromosomes (Table 5). Sixty-two percent were definitely in euchromatin, 24 percent were in heterochromatin, and 14 percent could not be distinguished.

Frequencies of dicentric breaks: The heterochromatic regions of the long arms are about equal in length. The dicentric breaks in each long arm occur with about equal frequency (Tables 2 and 4). This observation is substantiated by significantly low chi-square values (Table 6). The frequencies of dicentrics involving the short arms (Tables 2 and 4) also indicate a positive relation to heterochromatin. As indicated earlier, the entire short arms are heterochromatin and as shown in Table 6 the frequencies of breaks are also related to length.

Frequency of chromatid breaks

There were 1587 chromosome breaks and 35 chromatid breaks (Figure 5) in the various treatments. The chromatid breaks thus comprised 2.16 percent of the total breaks observed. These breaks appeared to be nonrandomly distributed, because 25 were in the long arm of D chromosome, six in the long arm of A, and four in the long arm of C. This distribution was not influenced by the oxygen treatments.

Frequency of intrahomologous exchanges leading to dicentrics

On the basis of the six Crepis chromosomes, 15 combinations or exchanges leading to dicentrics are possible. Of these, three (20 percent) can be exchanges

TABLE 6

Chi-square tests of the relation between breaks leading to dicentrics and length of heterochromatic regions in long and short arms of the chromosomes in X-irradiated Crepis capillaris *seeds*

	Observed	Expected	χ^2
Long arm of A	198	196	.020
Long arm of C	209	196	.863
Long arm of D	182	196	1.000
			1.883
Short arm of A	81	90	.900
Short arm of C	48	50	.080
Short arm of D	36	25	4.840*
			5.820

between homologous chromosomes. Table 7 shows that the number of intrahomologous dicentrics fits the expected value very well. The oxygen treatments did not influence the frequency of intrahomologous exchanges.

DISCUSSION

The present study clearly shows the pronounced after effect of oxygen treatment on X-ray induced chromosome aberration frequencies in seeds of *Crepis capillaris*. Thus, similar results found earlier in barley are confirmed (ADAMS, NILAN and GUNTHARDT 1955; NILAN 1955; ADAMS and NILAN 1958; CALDECOTT *et al.* 1957).

It is shown in the present study that an oxygen posttreatment does not influence differentially the induced chromosome breakage pattern in *Crepis capillaris*. Apparently oxygen is quite random in its influence and uniformly increases aberrations in all the chromosome arms.

Earlier reports (ADAMS, NILAN and GUNTHARDT 1955; ADAMS and NILAN 1958) have attempted to arrive at a suitable hypothesis to account for increased chromosome aberration frequencies during post-X-ray treatments. Two hy-

TABLE 7

Frequency of intrahomologous chromosome exchanges leading to dicentrics in X-irradiated seeds of Crepis capillaris

	Number	Percent
Total dicentrics	377	100
Observed intrahomologous dicentrics	87	23.1
Expected intrahomologous dicentrics	75.4	20.1
χ^2	1.78	..

potheses were considered appropriate. One of these concerned the effect of oxygen on the restitution of induced chromosome breaks (cf. SCHWARTZ 1952; SWANSON and SCHWARTZ 1953). The second relates to the effect of oxygen on the conversion of latent to primary breaks (cf. SWANSON 1954). However, from previous data it was impossible to distinguish between these two hypotheses.

Evidence has now been presented in this paper that suggests that a major effect of oxygen during the postirradiation treatments is to inhibit the restitution of chromosome breaks. As the storage period progresses, more and more breaks fail to restitute. Furthermore, many of the breaks do not rejoin to form dicentrics (Table 3). This is shown by the finding that the ratio of breaks that rejoined to those that did not rejoin decreased from 4.3:1 immediately after irradiation to 1:1 42 days after irradiation. This interpretation can account for the striking increase in fragments as compared to dicentric chromosomes found during oxygen storage of X-irradiated seeds.

Support for this interpretation can be found in the cytological studies of the after effects in barley seeds (ADAMS and NILAN 1958). In these seeds, also, there is a very marked increase of fragments during the postirradiation period and a much smaller increase of dicentric chromosome bridges. Furthermore, differential rejoining and restitution has been considered a factor in the increased chromosome sensitivity to X-irradiation in certain stages of mitosis in microspores of Tradescantia (BISHOP 1950; SPARROW and MALDAWER 1950; DESCHNER and SPARROW 1955).

In order to determine if the inhibition of restitution was due to oxygen or the pressure, an experiment was conducted using nitrogen as the storage atmosphere. Seeds in this experiment were irradiated at 1500r and stored separately under 100 lbs pressure of nitrogen (Industrial Air Products—99.6% N + 0.4% O_2 and CO_2) and 100 lbs pressure of oxygen for three weeks. The seeds were maintained at four percent moisture throughout the experiment.

It can be seen in Table 8 that the frequencies of aberrations after three week storage in nitrogen are somewhat higher than those in the nonstored seeds but much lower than those found in the seeds stored in oxygen. Presumably the slight increase in the nitrogen stored seeds is due to the oxygen already present in the seeds. Similar results have been obtained in this laboratory with barley

TABLE 8

Frequencies of abnormal cells, chromosome dicentrics, and rod and isodiametric fragments in X-irradiated (1,500r) seeds of Crepis capillaris *stored in oxygen and nitrogen at 100 lbs pressure from 0 to 21 days (summary of two experiments)*

Treatment		Cells		Chromosome aberrations	
X-ray	Posttreat.	Number	Abnormal percent	Dicent. /cell	Frag. /cell
1500r	no storage	276	12	.04	.16
1500r	N_2 storage	279	32	.10	.83
1500r	O_2 storage	306	86	.56	3.69

(ADAMS and NILAN 1958; NILAN and KRONSTAD 1958). Apparently, then, oxygen and not the pressure is chiefly responsible for the increase in aberrations and hence the inhibition of restitution in the X-rayed stored seeds.

Especially interesting is the finding that oxygen under pressure can induce chromosome aberrations (Table 1). Preliminary studies (unpublished) in barley have also indicated that oxygen *per se* can break chromosomes in seeds. This result supports the data of CONGER and FAIRCHILD (1952) which showed that oxygen can cause chromosome breaks in Tradescantia pollen.

A number of reports have presented evidence for non random chromosome breakage and many of these have been summarized by SPARROW (1951). NAVASHIN and GERASSIMOVA (1936) concluded that all the chromosomes of the set of *Crepis capillaris* broke with a frequency dependent upon their length. Their data, on the other hand, indicated that the frequency of chromosome breaks in the long arm of chromosome C was higher than would be expected on the basis of its length. This finding agrees with the results of the present work.

LEWITSKY and SIZOVA (1935) reported increased break frequency in the proximal regions of the long arms of chromosomes A, D, and C and in the central region of the long arm of C. They indicated that the "liability to fragmentation" in these arms is associated with a lesser contraction of the chromonema in these particular regions. However, these conclusions were reached from a small number of breaks, and the differences indicated may not be significant.

A large majority of the breaks that rejoin to form dicentrics are located in or near the heterochromatin. The breaks that do not rejoin (simple breaks), on the other hand, are most frequently found in the euchromatic regions (Table 5). This fact suggests that heterochromatin and euchromatin have different effects on chromosome rejoining and restitution.

The different influences of heterochromatin and euchromatin on the behavior of chromosome breaks can explain the apparent non randomness of the dicentric breaks in the long arms of A and C and of the simple breaks in the long arm of A and the short arms of C and D (Table 4). As the long arm of the A chromosome is the longest, one might have expected that it would have more dicentric breaks than the long arms of C or D. Such was not the case. Dicentric breaks occurred with equal frequency in the long arms of A, C, and D. Thus, though the breaks per chromosome arm were equal, breaks per unit of length were not. On this basis, the long arm of C had more breaks and the long arm of A fewer than expected. However, when compared by amounts of heterochromatin the observed breaks equalled the expected (Table 6). The frequencies of dicentric breaks in the short arms were proportionate to the lengths (Table 6). Here it should be recalled that the short arms are entirely heterochromatic.

The data pertaining to the simple breaks (Table 4) are not very extensive but nevertheless appear to fit into the general scheme. The increase of simple breaks over the expected in the long arm of A could be due to the longer region of euchromatin in relation to total length. Since the short arms of C and D are entirely heterochromatic no simple breaks would be expected and none were found. The

appreciable frequency of simple breaks in the short arm of A cannot be accounted for at this time.

The finding that breaks tend to rejoin more in heterochromatin than in euchromatin is difficult to understand. One clue may be obtained, however, from the reports that pairing between non-homologous chromosomes is initiated between heterochromatic segments in haploid rye (LEVAN 1942) and haploid *Triticum monococcum* (KOSTOFF 1938). Such an affinity of heterochromatin between non-homologous chromosomes of *Crepis capillaris* would possibly provide greater opportunity for rejoining between heterochromatic than between euchromatic segments of the chromosomes.

The data and above conclusions suggest that the apparent non randomness of chromosome breaks by X-rays in *Crepis capillaris* seeds is a consequence of the rejoining and restitution processes rather than the breakage process. In other words, the initial chromosome breakage in Crepis chromosomes is actually random, but through differential influence of euchromatin and heterochromatin on rejoining and restitution the breaks in certain arms appear to be non random. This hypothesis was suggested earlier by SAX (1940). SPARROW (1951), and SWANSON (1957). Furthermore, SPARROW (1951) summarized several reports which indicate that in chromosomes of certain species euchromatin and heterochromatin differ in their sensitivity to X-ray damage.

NAVASHIN and GERASSIMOVA (1936) reported that the X-ray induced intrahomologous exchanges in *Crepis capillaris* occurred at the expected frequency. The present paper supports this finding (Table 7) and further shows that the proportions of these exchanges are not altered by oxygen posttreatment.

The occurrence of chromatid breaks in supposedly monopartite chromosomes (cf. CALDECOTT and SMITH 1952) of resting seeds of *Crepis capillaris* cannot be accounted for at this time. Nor is there a ready interpretation for the high frequency of these breaks in the long arm of D.

CONCLUSION

From the above data and hypotheses it may be concluded that the initial X-ray induced breaks in chromosomes of resting seeds of *Crepis capillaris* are at random. The apparent non randomness of breaks by X-rays in certain arms of the chromosomes is due to the differential influence of euchromatin and heterochromatin on the rejoining and restitution of the chromosome breaks.

Oxygen applied after X-irradiation has no influence on the proportion of aberrations which occur in each chromosome arm. Nevertheless, through its influence on restitution and rejoining of chromosome breaks it uniformly increases the frequencies of chromosome aberrations in all arms.

During the oxygen posttreatments the broken ends in the euchromatin "heal," restitution and rejoining are inhibited, and an increasing number of fragments result. In the heterochromatin, on the other hand, the oxygen is not as effective in "healing" and some rejoining does occur. This can account for the much more rapid increase of fragments (from inhibition of restitution in the euchromatin)

than dicentric chromosomes (from some rejoining in the heterochromatin) during the postirradiation oxygen treatments.

SUMMARY

Resting seeds of *Crepis capillaris* (2n=6) were X-irradiated at 1500r or 3500r and then stored under 100 lbs oxygen pressure for periods up to 42 days. The frequencies of abnormal cells, dicentric chromosomes, and acentric rod and isodiametric fragments increased during the postirradiation oxygen treatment. Nonirradiated seeds exhibited an appreciable frequency of chromosome aberrations after 42 days of the oxygen treatment. Oxygen had no influence on the proportion of breaks in each chromosome because it uniformly increased the aberrations in all six chromosome arms. However, oxygen did appear to inhibit the restitution and rejoining processes since the ratio of breaks that did rejoin to those that did not rejoin decreased during the postirradiation treatment.

In both X-irradiated and stored X-irradiated seeds, certain arms exhibited frequencies of breaks leading to dicentric chromosomes and simple breaks (those that do not rejoin or restitute) that significantly differed from the frequencies expected on the basis of length of arms. The cause of this apparent non randomness was found in the relation of euchromatin and heterochromatin to chromosome breaks. Breaks leading to dicentrics occurred in or near the heterochromatic areas whereas the simple breaks occurred in the euchromatic areas of the chromosome arms. When the observed frequencies of breaks were compared with the lengths of heterochromatin and euchromatin in each arm, they equalled the expected frequencies. It is concluded that X-ray induced breakage probably occurs at random in *Crepis capillaris* chromosomes, and the apparent non randomness of these breaks is due to the differential influence of euchromatin and heterochromatin on the rejoining process.

The frequency of intrahomologous exchanges observed equalled the expected frequency. However, higher than expected frequencies of chromatid breaks were found; most of these breaks occurred in the long arm of D.

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CHEMICALLY-INDUCED INSTABILITIES IN A HETEROZYGOUS DIPLOID OF PENICILLIUM CHRYSOGENUM

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SOMATIC sergregation from heterozygous diploids in molds has been analyzed in detail for *Aspergillus nidulans* by PONTECORVO *et al.* (PONTECORVO, TARR GLOOR and FORBES 1954; PONTECORVO and KAFER 1956). Basically the same processes seem to take place in *Penicillium chrysogenum* (SERMONTI 1956, 1957), and probably also in *Aspergillus oryzae* (ISHITANI, IKEDA and SAKAGUCHI 1956). Somatic segregation analysis is comparatively straightforward where it concerns visible or easily selected markers (PONTECORVO and KAFER 1956; ROPER and KAFER 1957), but it becomes extremely laborious for invisible markers like nutritional deficiency, and impracticably difficult when the genes involved are those quantitatively controlling the production of antibiotics or other metabolites. Even segregation analysis of color markers is made laborious, in *Penicillium chrysogenum*, by the impossibility of microscopic identification of the color segregants as single yellow or white heads on the sporulating surface of heterozygous diploids (PONTECORVO 1953; SERMONTI 1957). Color segregants in this species appear, either as entire colonies or as macroscopic sectors, after plating conidia of the heterozygous diploid on complete medium. The rate of spontaneous appearance is sometimes so low that the collection of a modest number of segregants demands the observation of many thousands of colonies.

Attempts to increase the rate of somatic segregation of heterozygous diploids were made by IKEDA, ISHITANI and NAKAMURA (1957) on *Aspergillus oryzae* by ultraviolet irradiation of heterozygous diploid conidia, and by MORPURGO and SERMONTI (1958) on *Penicillium chrysogenum* by ultraviolet irradiation of conidia and by treating them with various chemical mutagenic agents. Somatic segregation was strongly stimulated in almost every case by the action of the mutagenic agents. In MORPURGO and SERMONTI's paper (1958), the delayed appearance of segregating sectors in colonies derived from conidia which had survived mutagenic treatment was very striking, suggesting a delayed effect of the mutagenic agent. An analogous delayed effect of chemical mutagenic agents had been noticed by AUERBACH (1951) in *Drosophila melanogaster*.

The present paper was begun as an analysis of this effect in the material under discussion.

MATERIALS AND METHODS

Strain: The strain used throughout the work was heterozygous diploid XXXIV of *Penicillium chrysogenum* from the Istituto Superiore di Sanità collection. The

diploid was synthesized by ROPER's method (ROPER 1952; PONTECORVO and SERMONTI 1953) between strains *173 y py* (derived from strain Wis. 49.133) and *176 w cy* (derived from strain Wis. 50.1247). The genotype of heterozygous diploid XXXIV may therefore be symbolized as:

$$\frac{Y\ PY\ w\ cy}{y\ py\ W\ CY}$$

No information was available at the beginning of the experiments about the linkage relations between the markers concerned. An attempted mapping will be discussed in the last section on the basis of the results reported. The symbols used are to be understood as follows : Y/y, green *versus* yellow conidium color; PY/py, nutritional independence *versus* requirement of pyridoxin; w/W, white *versus* green condium color; cy/CY, requirement of methionine or cysteine *versus* nutritional independence. The phenotype of the diploid is prototrophic and has green conidia.

Use of nitrogen mustard: The only agent used for stimulation of somatic segregation was a pharmaceutical preparation of the nitrogen mustard methyl-bis(β-chloroethyl)amine (Cloramin) in sterile 5 mg vials (SIMES, MILAN). It will be referred to hereinafter by the code name HN-2. Two ml of a sterile aqueous solution of $NaHCO_3$ (6.3 percent, w/v) were added with a Pasteur pipette to one vial of HN-2, shaken until the mustard was dissolved and then mixed with two ml of an aqueous suspension of 4.10^7 conidia of the strain to be treated. After 3–4 minutes the suspension was diluted ten times in a sterile decontaminating solution (0.6 g glycine, 0.7 g $NaHCO_3$, one liter distilled water.) The decontaminated suspension was kept at +5°C until required for use. The survival rate of the conidia at the end of treatment was 0.008–0.050, with no appreciable variation during the first 3–4 weeks of storing at +5°C.

In the last part of the experiments the method was modified; the HN-2 was dissolved in distilled water, and the conidia were suspended in the aqueous solution of $NaHCO_3$. Only in a very few cases was ultraviolet irradiation used as a mutagenic agent (PONTECORVO and SERMONTI 1954).

Media: The media used were a complete medium (CM), based on corn steep-liquor, nucleic acid, vitamins, etc. (SERMONTI 1957), and Czapek-Dox's minimal medium (MM).

Plating of conidia: The method adopted as standard in this laboratory was used in the first part of the experiments, i.e. 0.1 ml of conidium suspension of known density was spread with a glass rod over the agar surface of each petri dish. A less cumbersome method was later employed for carrying out platings starting from a single colony: conidia are harvested from the surface of the colony with a small drop of water held in a calibrated loop, which is then shaken in 2 ml of sterile water. The suspension is then diluted 1,000 times. Suspensions obtained in this way contain 10–30 conidia per 0.1 ml and can be plated by the standard method. *Penicillium chrysogenum* conidium chains immediately break up when placed

in water and gently shaken, so that the conidium suspensions always consisted of isolated conidia. Cultures were incubated at 24–27°C.

Terminology: Single colonies presumably developed from single conidia will be regarded as individuals in this paper, with a life cycle extending from germination of the original conidium to conidium production in the colony derived from it. Colonies derived from plating of the latter conidia will be regarded as belonging to the "generation" following that of the colony producing such conidia. This convention simplifies the record of the life history of colonies, by simply mentioning the "generation" to which they belong. Colonies obtained from conidia of heterozygous diploid XXXIV will be regarded as belonging to the parental generation (P), those from conidia of the P colonies as belonging to the first filial generation (F_1). Later generations will be recorded as F_2, F_3, etc. This terminology is purely conventional.

RESULTS

Effect of HN-2 on somatic segregation

HN-2-treated and untreated (control) diploid conidia were plated on complete agar at density of about 30 living conidia per dish. The colonies were observed for about ten days and on the last day of observation the number of colonies segregating for color or showing segregant sectors was recorded. The proportion of colonies displaying markers heterozygous in diploid XXXIV was greatly increased by the action of HN-2 on the conidia (Table 1). An analogous effect has

TABLE 1

Somatic segregation from diploid XXXIV: y py / w cy *of* Penicillium chrysogenum: *spontaneous segregation and segregation after HN–2 treatment*

Phenotype	Symbol	Number of segregants observed		
		Whole colonies	Sectors	Total
A. Spontaneous segregants				
Yellow prototrophic	*y*	10	7	17
pyridoxineless	*y py*	..	..	..
White prototrophic	*w*	..	..	..
cysteineless	*w cy*	..	1	1
cyst.-pyrid.-less	*w cy py*	..	..	..
Green (non tested)		706	7	713
B. Segregants after treatment				
Yellow prototrophic*	*y*	11	16	27
pyridoxineless†	*y py*	5	2	7
White prototrophic	*w*	5	..	5
cysteineless‡	*w cy*	22	6	28
cyst.-pyrid.-less‡	*w cy py*	1	2	3
Green prototrophic	+	141	21	162
pyridoxineless	*py*	2	1	3

* Conidia sometimes of haploid size, sometimes of diploid size.
† Conidia always of haploid size.
‡ Among 14 white segregants from another clone, 7 were *w cy* and 7 were *w cy py*.

been obtained with diploid VII (*me w/y thi pr*) (results not published in this paper). What is particularly striking is the increased proportion, among colonies derived from conidia surviving HN-2 treatment, of colonies exibiting segregation of color markers in the form of sectors. In such sectors the segregation process is certainly subsequent to the plating of the conidia. The rate of such sectoring colonies among untreated colonies is about 0.01, while among treated colonies it is about 0.1 (Table 1). The proportion of complete yellow or white colonies is also increased after HN-2 treatment, from 0.014 to 0.18 (Table 1). These could have been produced by a segregation process previous to conidium formation, but the great increase in their number among colonies derived from treated conidia makes it highly probable that in this case the majority of the segregant clones have originated subsequent to conidium collection, as a result of the action of HN-2. If it is assumed that each segregation process gives rise to two segregant nuclei in such cases one of the segregant nuclei must be supposed to have been eliminated, either by a disadvantageous mutation or as a result of genetic drift. Alternatively, some segregation process must be assumed which does not give rise to complementary nuclei, such as deletion, aneuploidization, etc.

Seven different segregant phenotypes out of the ten theoretically possible appeared after HN-2 treatment. Their ploidy is not easy to determine, on account of the irregularity of conidium size within each strain. Some of the yellow colonies (see note to Table 1) produced what were clearly normal conidia of the same size as those of haploid strains, while others produced giant conidia of the same size as those of diploid XXXIV. Giant conidia were also produced by green pyridoxinless (*py*) segregants. An analysis of the second-order somatic segregation from one of these *py* strains, purified by isolation of a single conidium, showed that the strain was heterozygous for the genes Y/y, W/w, and CY/cy.

The number of sectoring colonies among colonies derived from mustard-treated conidia increased as time went on, with the delayed appearance of sectors from some colonies. This phenomenon invited special consideration. It could be attributed to the delayed emergence of a segregant nucleus in the course of growth of a colony; but it could also be due to a delayed effect of the mutagenic agent. This assertion was tested by taking green conidia from a number of color sectoring colonies and plating them on CM in order to observe the appearance of the colonies derived from them. These colonies will be regarded as the first filial generation (F_1) of the sectoring colonies.

Analysis of F_1 of sectoring colonies

A total of 28 colonies with sectors, derived from HN-2-treated conidia, were analyzed by the procedure indicated above. Colonies of the first filial generation (F_1) were observed for about ten days of growth on CM; the proportion of colonies with sectors among them, and the type of each colony, was recorded. Hardly any completely yellow or white colonies were found.

Seventeen of the F_1 populations of colonies examined showed a normal segregation rate, i.e. one not significantly different from that of diploid XXXIV. Three

showed an appreciably higher proportion of sector-producing colonies, but the other eight showed a feature which had never previously been observed: the majority of the colonies, in a few cases almost all of them, produced sectors (Figures 1–4). Clones, or lines, derived from sectoring colonies, in which the property of producing sectors persisted in successive generations, will be referred to by capital letters (A, B, C, etc.). The term "mosaic" colonies will sometimes be used to refer to sectoring colonies, and the term "mosaic" clones, or lines, for the clones, or lines, to which they belong.

The proportion of mosaic colonies in the F_1 of clones with a high rate of segregation varied from 30 percent (D) to 90 percent (G). A particularly striking feature of these populations of colonies with a high rate of segregation was the uniformity of segregation pattern within each clonal population. In the majority, the sectors which appeared were exclusively yellow; in two clones (B and G) the sectors were of both colors, yellow and white, and a fair proportion of the colonies had sectors of both colors. One clone had colonies with white sectors only (L) and one had pale yellow and grey-green sectors in the majority of the colonies (C) (Table 2). The type and size of the sector was also characteristic in

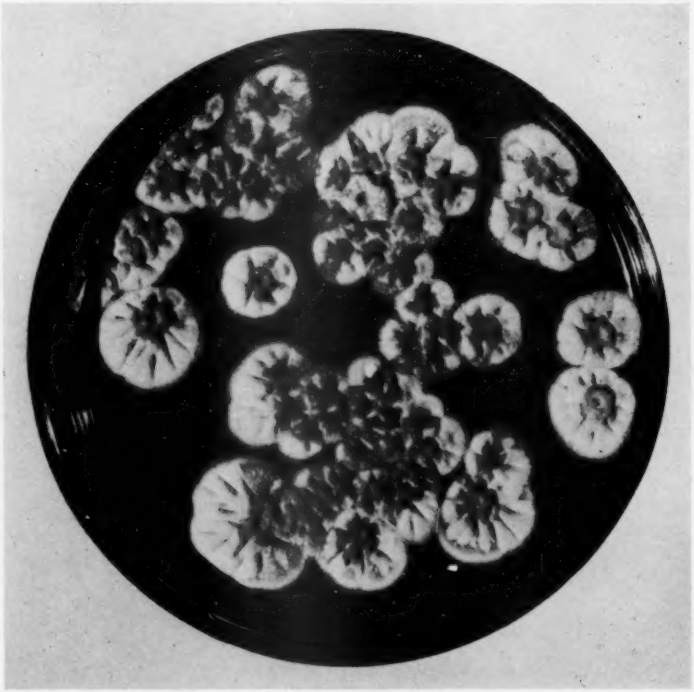

FIGURE 1.—Colonies of the unstable line D, showing yellow zones.

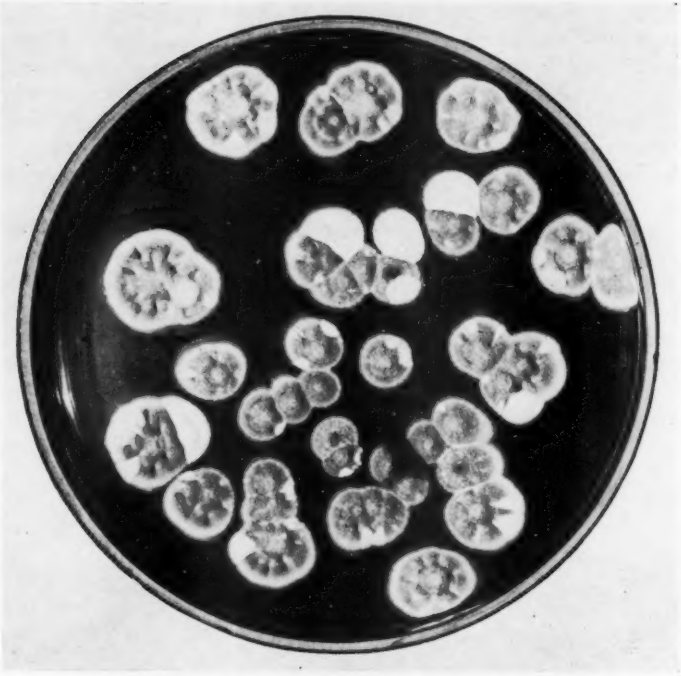

FIGURE 2.—Colonies of the unstable line N, showing white sectors.

TABLE 2

Clonal populations with a high proportion of mosaic colonies, derived from conidia of unstable lines (diploid XXXIV: y py / w cy)

Unstable line (code)	Number of colonies obtained after plating conidia						Sector characterization			
	Colonies showing sectors			Colonies without sectors (no.)	Colonies unclassified (no.)	Totals (no.)	White		Yellow	
	white (no.)	yellow (no.)	white & yellow (no.)				Tested (no.)	Phenotype*	Tested (no.)	Phenotype*
A	0	59	0	5	6	70	..	....	36	*y*
B	15	192	220	42	32	501	28	*w cy*	52	*y (py)*
C	0	120	0	5	3	128	..	....	59	*y*
D	0	69	0	5	4	78	..	....	27	*y*
F	0	41	0	5	0	46	..	....	51	*y*
G	3	50	23	8	1	85	26	*w cy*	68	*y (py)*
H	0	32	0	38	0	70	..	....	22	*y cy*
L	11	0	0	0	14	25	11	*w*	..	..
M	0	13	0	26	2	41	..	....	27	*y*
N	123	0	0	0	161	284	100	*w cy*	..	..

* The allele symbols indicate the phenotype.

each clone. In some populations the segregant zone of the colony was so large that there was only a small central green zone, surrounded by the yellow or yellow-and-white remainder of the colony (Figure 1). The opposite extreme was reached in one population (H), in which the segregant zones appeared as insignificant spots, and in another (E), in which only small sectors appeared, very late and at the extreme edge of the colonies. Clone M, which appeared spontaneously, produced late grey-green sectors, with a few small yellow spots which turned out to be prototrophic (Figure 4).

Three green colonies derived from HN-2-treated conidia were subjected to analysis, and in each case F_1 showed normal color segregation rates.

Ten colonies with sectors derived from untreated conidia of diploid XXXIV were similarly analyzed. Only one of these (M) showed a very high proportion of yellow sector-producing colonies in F_1 (13/41). The sectors were of small size and appeared within a wide grey-green zone protruding from the edge of the colony (Figure 4).

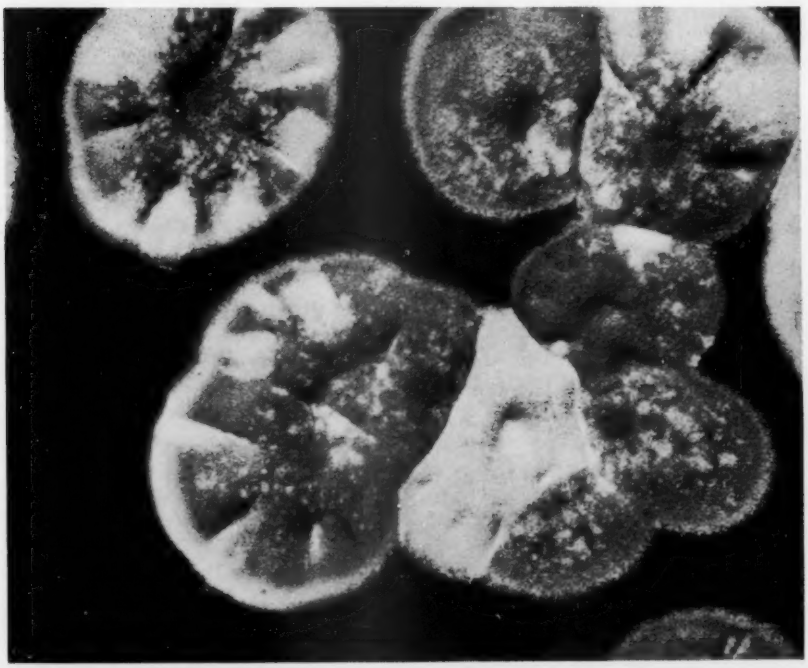

FIGURE 3.—Colonies of the unstable line F, showing yellow sectors and spots.

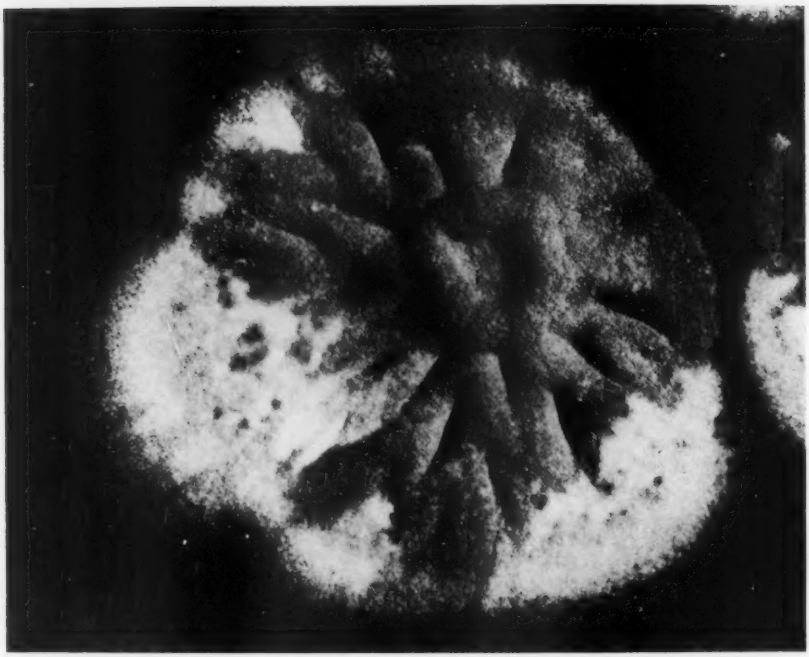

FIGURE 4.—Colonies of the spontaneous unstable line M, showing grey sectors with yellow spots.

Analysis of further generations

A number of F_1 colonies were used for an analysis of the next generation. Plating methods and determinations of the proportion of mosaic colonies in the populations studied were the same as those described in earlier sections.

Eleven sectoring F_1 colonies derived from mosaic clones were studied, and all eleven gave an F_2 with a high proportion of mosaic colonies. All the clones concerned were derived from single HN-2-treated conidia. The mosaic pattern of the F_2 colonies in the various clones was the same in each case as that displayed by the F_1 colonies.

Four different mosaic colonies of the F_1 of clone B (yellow and white sectors) were examined, one with yellow and white sectors only, and two with yellow sectors only. All four produced an F_2 with a high proportion of yellow and white sectors. Moreover, one colony without sectors of the F_1 of clone B (see next section) produced an F_2 with a high proportion of colonies with white and yellow sectors and sectors in both colors. The F_1 colony which had a white sector only gave a higher proportion of white sectoring colonies in the F_2 (0.37) as compared with the two F_1 colonies with yellow sectors (0.16 and 0.18).

Three sectoring colonies of the F_2 of mosaic clones were used to study the F_3; in each case the F_3 populations reproduced the high segregation rate and the mosaic pattern of the clone to which they belonged.

The rates of mosaic colonies in successive generations of several mosaic clones have been recorded. It has been noticed that there is an appreciable increase in segregation rate as the generations proceed. Clone D, for instance, gave 30 percent mosaic colonies in F_1, 45 percent in F_2, 100 percent in F_3 (Figure 1). It should be mentioned that the colonies used for the production of conidia of the next generation were not picked out at random, but chosen from those showing the mosaic configuration.

From the results obtained up to the present it appears that the tendency to produce progeny with a high proportion of mosaic colonies behaves as a hereditary character in the clones analyzed. This character manifests itself in single colonies by the appearance of segregant zones or sectors. The character in question will be referred to by the symbol HSF (high segregation frequency).

Reversion of character HSF in some colonies.

In almost all the HSF populations a certain number of colonies appeared which were completely green, i.e. without the mosaic appearance characteristic of the clone. Three of these colonies, belonging to the F_1 of clone B, were used for an analysis of their respective offspring. One of these (see above section) gave rise to an HSF population, although with a relatively low proportion of mosaic colonies (85/195), and two gave rise to populations with normal segregation rates (0/144 and 1/183). A colony without sectors obtained from line F also showed an almost normal proportion of mosaic colonies in the next generation (5/176).

Although the colonies examined represent only a very small sample, it is clear that the presence of green colonies is due partly to incomplete penetrance of the character HSF and partly to reversion of the character to the normal state. The loss of capacity to produce mosaic offspring does not depend on the occurrence of homozygosis at the locus of the segregant markers, since they continue to appear in the offspring, although very sparsely (Table 3, Clone F).

By repeated transfer under uncontrolled conditions, an unstable strain may lose its characteristic instability, probably because of selection of reverted subclones. This happened with line L; practically no colonies with white sectors were longer detectable after a few transfers. To restore the line an occasional white sectoring colony was selected, which initiated the unstable line N (Figure 2). The white sectors produced by the colonies of the latter line, instead of being prototrophic as the white sectors from the clone L, were regularly cysteineless (Table 2).

Characterization of segregants

From mosaic colonies of some of the HSF clones a few color segregant zones were collected and tested for nutritional requirements. The zones were touched

TABLE 3

Exceptional segregants from conidia of some unstable lines, appearing after mutagenic treatments (diploid XXXIV: y py / w cy)

Unstable line		Exceptional segregants after mutagenic treatment						
			Selected for color			Selected for requirements		
				Segregants			Segregants	
Code	Spontaneous sectors (phenotype)*	Mutagenic agent	Colonies observed (no.)	(No.)	(Phenotype)*	Colonies tested (no.)	(No.)	(Phenotype)*
A	*y*	u.v.	350	..	..	208	1	*py*
C	*y*	HN–2	3360	..	..	...	..	..
D	*y*	HN–2	859	6	*w*	...	..	
F	*y*	HN–2	740	2	*w*	...	..	..
		u.v.	1104	1	*w*	...	..	..
F†	..	....	176	5	*y*	...	..	..
		u.v.	1360	39	*y*	...	..	..
				1	*w py*	...	..	..
				10	*w*	...	..	..
				1	*w cy*	...	..	..
H	*y cy*	u.v.	602	..	..	120	2	*y*
							1	*y cy py*
L	*w*	HN–2	576	..	..	45	8	*w cy*

* The allele symbols indicate the phenotype.
† From a reverted non-sectoring colony.

with the point of a needle which was then streaked on to complete agar; streaks which gave rise to growth of homogeneous colour were later tested for nutritional requirements, while those which turned out not to be pure were further purified and then tested. The test consisted of transplating conidia of the purified segregant clones on to MM, MM supplemented with pyridoxine (0.07 mg/ml), MM supplemented with methionine (0.05 mg/ml), and in some cases MM supplemented with both compounds.

The right-hand side of Table 2 shows the phenotype of a number of segregants belonging to various clones. It was observed that each mosaic clone usually gives rise to only one colored phenotype (in the case of lines B and G two colored segregant types were observed). Within each clone the colonies of the same color all have the same nutritional requirement. Thus each mosaic clone is uniform within itself in respect not only to the color of the segregant sectors and to their size and shape but also to their nutritional requirement.

Clear distinctions can be drawn between at least six different mosaic patterns. Two clones (B and G) produce large white sectors with cysteine requirement, and large yellow sectors with a pyridoxine requirement; the yellow segregants show slight growth even in the absence of pyridoxine, unlike the component strain *y py* and some other pyridoxine-deficient segregants which show an absolute pyridoxine requirement. One clone (C) produces wide yellow prototrophic and grey-green prototrophic sectors; two clones (D and F) produce wide yellow prototrophic sectors (Figures 1 and 3); one clone (H) produces small yellow

spots with a cysteine/methionine requirement, i.e., a recombinant phenotype; one clone (L) produces wide white prototrophic sectors; one clone (N) produces sharp white cysteineless sectors (Figure 2).

In the majority of the mosaic lines the rate of growth of the isolated sectors was greater than that of the green zone of the colonies. The opposite holds for line H, in which the rate of growth of the isolated sectors was much lower than that of the green zone of the colonies.

No reliable data could be obtained about the conidium size of the mosaic colonies and their segregant sectors, on account of the great variability in shape and size of the conidia of different strains. This was particularly noticeable in the conidia of some segregant sectors. Anyway the conidium size from the nonsegregating part of the unstable colonies is as a rule diploid, while that of the segregant sectors is considerably more irregular but in some cases decisively diploid.

Exceptional segregants from mosaic clones

In order to determine whether the mosaic clones were still heterozygous for the genes for which they did not show spontaneous segregation, green conidia of a number of colonies of various mosaic lines were treated in such a way as to stimulate a somatic segregation.

The details of these experiments are shown in Table 3. The general result was the detection in many HSF clones of markers which showed no appreciable spontaneous segregation. Particularly noticeable were: the occurrence of several white segregants in clone D, which spontaneously gave only yellow segregants; the occurrence of two yellow prototrophic segregants and one yellow cysteine-pyridoxine-deficient segregant in clone H, which spontaneously gave only yellow cysteineless segregants; and the occurrence of white cysteineless segregants in clone L, alongside the spontaneously occurring white prototrophic segregants.

The induced segregation in clone F calls for special mention. The spontaneous segregants in this clone were yellow; some white segregants occurred after HN-2 treatment or ultraviolet irradiation of the conidia. A nonmosaic colony of clone F was also examined, in the filial generation of which occasional yellow sectoring colonies appeared spontaneously (5/176). After conidia of this nonmosaic colony had been subjected to ultraviolet irradiation, a few white segregants appeared alongside the yellow segregants, in a proportion (12/1360) noticeably higher than that (1/1104) in which such segregants appeared after treatment of conidia of a mosaic colony of the same clone F.

DISCUSSION

Methyl-bis(β-chloroethyl)amine (HN-2) turns out to be an effective agent for stimulating somatic segregation in diploids of *Penicillium chrysogenum*, as was already observed by MORPURGO and SERMONTI (1958). The chemical treatment increased the proportion of segregant colonies or colonies with segregant sectors from 0.023 to 0.340 in the experiments recorded here. Practically all the detectable segregants are the result of treatment with the mutagenic agent. This fact

is of particular interest because each segregant obtained after stimulation is the result of an independent chromosomal rearrangement, whereas among the colonies obtained from untreated conidia two segregants of the same phenotype may belong a single segregating clone, originating before conidium formation. This circumstance facilitates genetic analysis of diploids of *Penicillium chrysogenum*, which is, on the other side, simplified by the very high rate of appearance of segregants. Difficulties are of course created by the impossibility at present of stating which particular chromosomal rearrangements are produced by mutagenic treatment of conidia. There is no need to dwell on the practical importance of mutagenic treatment for the purposes of industrial ultilization of the parasexual process. The genetic variability of a population of colonies derived from treated heterozygous diploid conidia is enormously increased, and the breeder can have at his disposal a much greater variety of phenotypes.

A large part of the induced segregants take the form of apparently homogeneous colonies. The remainder appear as yellow or white sectors from green colonies. In about 40 percent of cases, the green part of the colonies gives rise to unstable lines (HSF = high segregation frequency), which are discussed below. In the rest of sector-producing colonies the green part breeds true (not-HSF). Since the number of not-HSF sectoring colonies greatly increased after HN-2 action, in these cases we must assume a delayed effect of the mutagenic agent.

The increased proportion of sectors in colonies of the unstable lines may be explained in two different, but not mutually exclusive, ways. On the one hand, it may be due to a selective advantage enjoyed by the segregant clone as compared with the HSF clone from which it emerges as a sector. In this case there is no need to assume, in the mosaic lines, any increase in the rate of occurrence of the processes which lead to somatic segregation : the increased proportion of sectors could be explained by a normal rate of occurrence of segregation processes with a greater likelihood of sector emergence in the segregant clones. The other possibility is an actual increase in the rate of occurrence of the segregation processes in the mosaic lines.

A model representing the first condition is the occurrence of a dominant semilethal mutation on the chromosome homologous to the one carrying the markers with a high rate of segregation. Segregation of the wild type allele of the semilethal gene would involve segregation of markers linked to it, and possibly of other markers as well, and would give a selective advantage to the segregant clone.

An analogous situation occurs in heterozygous acr_1/ACR_1 diploids of *Aspergillus nidulans* (Roper and Kafer 1957) in the presence of acriflavine. The semidominant allele acr_1 (sensitivity to acriflavine) is harmful to the heterozygous diploid. It is eliminated by haploidization (giving ACR_1) or by somatic crossing-over between this allele and the centromere (giving ACR_1/ACR_1). The derived clones emerge in the form of vigorous sectors from heterozygous colonies of stunted growth. Haploidization leads to segregation of the markers linked to ACR_1, and sometimes to segregation of markers lying on other chromosomes.

Somatic crossing over leads to segregation of the markers distal to ACR_1 and sometimes to segregation of some of the markers lying between ACR_1 and the centromere.

The details of the linkage relations of the markers in diploid XXXIV are still far from completely known, but on the basis of criteria formulated in an earlier paper by one of the present authors (SERMONTI 1957), *py* appears to lie on a different chromosome from that (or those) carrying *y*, *w* and *cy*; *w* and *cy* seem to be on the same chromosome arm with *w* distal to *cy*. Whatever the linkage situation, the elimination of a harmful gene by somatic crossing over or haploidization should give rise to a variety of *noncomplementary* phenotypes, some markers being automatically selected while others are free. No such state of affairs was observed in any of the lines analyzed (see Table 2), and this cannot be attributed to the loss in the HSF clones, of the nonsegregant markers, since in many cases (see Table 3) these turned out to be present in the heterozygous state. Moreover, although better growth of the isolated sectors, as compared with the colonies of origin, was actually observed in many lines, the growth of the isolated sectors in line H was less vigorous than that of the colonies of origin.

A second model may be adduced, again from *Aspergillus nidulans*, to exemplify the first condition. Loss of a complete chromosome behaves as a semidominant disadvantageous mutation; the aneuploid strains grow weakly due to a kind of unbalance (PONTECORVO and KAFER 1957; KAFER 1957). They throw out vigorous sectors which rapidly overwhelm the poorly growing seed colony. Repeated isolation of spores of the strain of the colony usually produces the same configuration. The conidial size from the vigorously growing sectors is haploid, conidia from the center are variable, and range from almost diploid to haploid. It should be observed that, theoretically speaking, aneuploidy could produce, alongside of a selective disadvantage, an actual increase in the rate of occurrence of the process of haploidization, due to some inherent irregularity in the mitotic process.

This hypothesis is open to the same objections as the semilethal-mutation hypotheis: a variety of segregant phenotypes should be expected within each mosaic clone. Besides, the situation with respect to conidium size of the unstable clones is somewhat different from that which appears with aneuploid clones in *Aspergillus nidulans*.

The second explanation of the increased proportion of sectors in the unstable lines is an actual increase in the rate of occurrence of segregation processes. This hypothesis rests on the exclusion of explanations based on selective effects. Although not yet critically proved, there seems at present to be more support for the assumption of an increase in the rate of segregation as an explanation of the phenomena observed.

The occurrence of genetic instability in the material described in this paper is certainly due to a mutational event. The character "Instability" (HSF) is strictly hereditary, its appearance is sudden, and its rate is greatly increased by treatment with mutagenic agents. A small proportion (1:10) of the colonies which produced sectors spontaneously in diploid XXXIV originated unstable lines and

since colonies which produce sectors for color appear with a frequency of approximately 0.01 (7:723), the absolute frequency of colonies producing unstable clones is of the order of 10^{-3}. After HN-2 treatment, mosaic colonies represented about ten percent (23:208) of the surviving colonies, and about 40 percent (11:28) of these were unstable. The absolute proportion was therefore of the order of 4.10^{-2}, an increase of about 40 times over the spontaneous rate.

The results reported here give a strong indication that the induced instability affected different chromosomal regions in the different mosaic clones. Indeed, there is a remarkable specificity: each unstable line is unstable for particular markers only, and at least six different instability patterns have been identified in the nine lines examined. There is high specificity also in the time of appearance and the shape of the sectors in different lines. One could suppose that the various clones are differentiated by the disappearance before sector production of certain markers in some of them, and that the instability itself may be generic, appearing specific only because each clone is only able to segregate for markers which have remained in the heterozygous state. This has turned out not to be the case, since the heterozygosis has been demonstrated in the clones analyzed of some markers which nevertheless did not segregate with high frequency.

The exact nature of the processes of chromosomal rearrangement which give rise to mosaic clones cannot be determined with any certainty in the present state of knowledge of the genetic make-up of the diploid concerned. The existence of clones producing sectors of two complementary types suggests that, in such cases, either the process of somatic crossing over in one or more particular regions or the segregation of whole chromosomes has been stimulated.

It is unfortunate that irregular conidial size of the segregant sectors does not allow of their ploidy classification.

A large number of cases of genetic instability have been observed in maize (McCLINTOCK 1951). In some of these a locus, *Ds.* has been identified which displays a marked fragility. When a rupture occurs at this locus the acentric fragment produced is eliminated during mitotic division. All the dominant alleles in the fragment are removed and any recessive alleles present in the corresponding segment are thus enabled to express themselves in the phenotype. Grains during the development of which such phenomena have occurred are variegated.

This model is more closely applicable than any other as an explanation of the instability in *Penicillium chrysogenum*, in cases in which the segregation concerns a single phenotype.

Cases of radiation-induced genetic instability were observed by NEWCOMBE (1953) in Streptomyces sp. The phenomenon has many points of similarity with the present observations. NEWCOMBE (1953) thinks that the mechanism responsible for the development of unstable lines in maize (McCLINTOCK 1951) might be applicable also to Streptomyces. Unlike the material studied here, the strain from which NEWCOMBE (1953) obtained unstable lines was assumed to be haploid.

Rupture points, with loss of chromosomal fragments, have been suggested in

Escherichia coli K12 diploid (NELSON and LEDERBERG 1954) and haploid (CAVALLI and JINKS 1956).

In 1925 BRIDGES observed, in females of *Drosophila melanogaster* containing the dominant factor Minute-n on one of the X-chromosomes and certain recessive genes on the other X-chromosome, a frequently occurring mosaic condition, i.e. the appearance of small areas in different parts of the body which were not phenotypically Minute-n and expressed the effects of the recessive genes. BRIDGES suggested that the factor Minute-n had the property of occasionally eliminating the X chromosome on which it lay.

STERN (1936) showed that the chromosome elimination theory was not satisfactory as an explanation of the mosaic formation and that the mechanism of the phenomenon in question rests *primarily* on the occurrence of somatic crossing over. The increase in the rate of somatic crossing over must depend on a general "phenotypic Minute reaction" on development.

Hereditary tendencies to delection, loss of complete chromosomes, and somatic crossing over may all play a part in the phenomenon of genetic instability in *Penicillium chrysogenum*, while selective effects probably take part only in so far as they determine the morphological pattern of the mosaic colonies.

SUMMARY

The action of methyl-bis(β-chloroethyl)amine (HN-2) on the somatic segregation of a diploid strain of *Penicillium chrysogenum* heterozygous for color and deficiency markers is studied. The following points are established:

1) HN-2 greatly increase the proportion of segregants observable as whole colonies or as yellow or white sectors from green colonies.

2) About a third of the colonies with sectors ("mosaic colonies") derived from treated conidia gave unstable clones after repeated transfer of the green zone. This instability appears as a capactiy of the conidia to produce a very high proportion of mosaic colonies. The instability persists for an indefinite number of generations.

3) One unstable clone was derived from a mosaic colony which had formed spontaneously.

4) Absolute specificity of segregation pattern was shown by the unstable clones: each clone produces only one or two types of segregant sector. However, tests carried out on clones showed that markers not concerned in the high frequency were present in the heterozygous state.

5) Reverted stable subclones, in which the markers showing a high segregation rate were still present in heterozygous state, were readily obtained from the unstable clones.

6) Possible causes of the instability are discussed. An actual increase in the rate of occurrence of somatic segregations seems to be responsible.

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